Caracterización biológica y pronóstica del linfoma difuso de células grandes en la era de la inmunoquimioterapia

Gonzalo Gutiérrez-García

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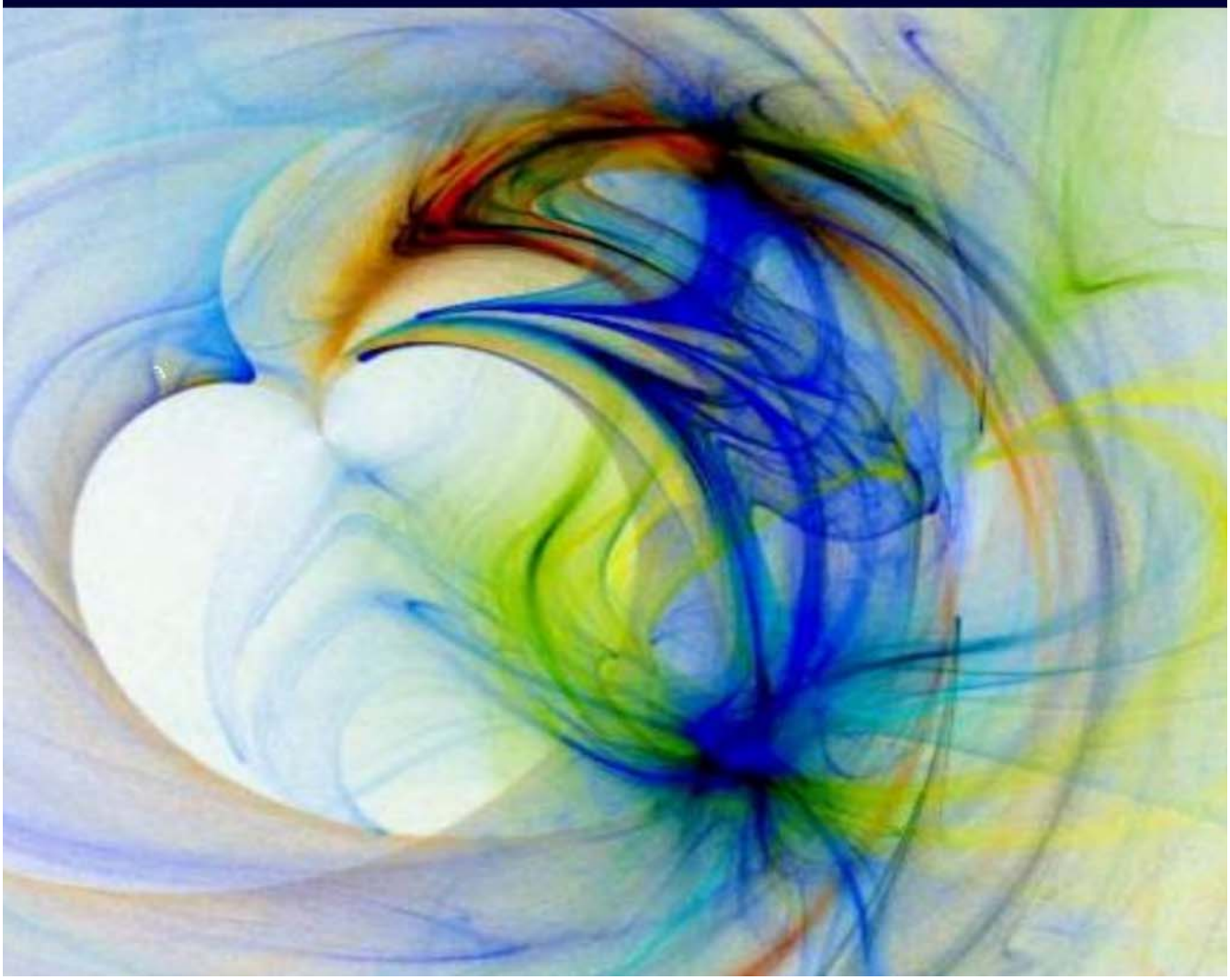

CARACTERIZACIÓN BIOLÓGICA Y PRONÓSTICA DEL LINFOMA DIFUSO DE CÉLULAS GRANDES EN LA ERA DE LA INMUNOQUIMIOTERAPIA

Tesis presentada por

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Para optar al grado de Doctor en Medicina

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A Dios, que es el centro de mi vida

A mí madre, Victoria Carmen Rosa que te debo todo lo que soy

A mis hermanos Liliana y Miguel Ángel, la misma esencia en diferente forma

A Jaditu por cada instante

Yo soy el Alfa y la Omega, principio y fin
Apocalipsis 1:8

Para mí, es "realmente" difícil hacer un resumen de casi ocho años en el que sin lugar a dudas es "El mejor Servicio de Hematología del Sur de Europa". Durante este tiempo, han pasado muchas cosas buenas y malas (más de las buenas), eso sí, todas ellas enriquecedoras. Llegué al hospital con grandes expectativas, inmenso entusiasmo, profunda timidez, y sobre todo con un gran desconocimiento de prácticamente todo. La mejor lección trasmitida por todos vosotros se llama **Estructura**, es aquí donde está la clave que identifica la denominación de origen "Clínic".

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permitido tener una vida más agradable en el hospital: Alejandra (amiga y cómplice), Silvia, Gabriela, Eukene, Jordina, Xavi y Carlos. Gracias a todos por los buenos momentos.

Por último, quiero agradecer a todos aquellos que han contribuido a mi formación tanto profesional como personal a lo largo de estos años. A todos vosotros, gracias.

Gonzalo Gutiérrez García
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Contenido

Abreviaturas

ABC: activado

CGB: centrogerminal B

ECOG: Eastern Cooperative Oncology Group

EG: extraganglionar

Igs: inmunoglobulinas

IMiDs: immune-mediated inflammatory diseases

IPI: Índice Pronóstico Internacional

IQT-R: inmunoquimioterapia con rituximab

LDCGB: linfoma difuso de células grandes B

LDH: lactato deshidrogenasa

LF: linfoma folicular

LH: linfoma Hodgkin

LLC: leucemia linfática crónica

LNH: linfoma no-Hodgkin

MALT: mucosa-associated lymphoid tissue

No-CGB: no centrogerminal B

NOS: not otherwise specified

R-IPI: Índice Pronóstico Revisado

RC: remisión completa

SG: supervivencia global

SLP: supervivencia libre de progresión

SNC: sistema nervioso central

I. INTRODUCCIÓN

Introducción

1. Linfoma difuso de células grandes B (LDCGB): concepto, clínica, pronóstico y tratamiento

1.1. Concepto y clasificación

El LDCGB es el linfoma más frecuente en el adulto y el paradigma del linfoma agresivo.(1) La incidencia en España es de 30.000 nuevos casos cada año y su prevalencia oscila entre 30 y 50% de todos los linfomas no-Hodgkin (LNH).(2)

El concepto de LDCGB ha cambiado sustancialmente a lo largo de los últimos cincuenta años, de modo similar a como ha evolucionado el sistema clasificatorio de las neoplasias linfoides. (Tabla 1) La clasificación de Rappaport (1966) puede considerarse como la primera clasificación moderna y se basaba exclusivamente en la arquitectura y morfología del ganglio linfático.(3) Posteriormente, la clasificación de Kiel y la de Luke-Collins (1974) incorporaron las características histológicas y el tipo celular.(4) Durante los siguientes años hubo una inflación de clasificaciones a ambos lados del Atlántico, con grandes dificultades para reproducir y aplicar los diferentes sistema clasificatorios. Sistemáticamente, las clasificaciones de Rappaport y Luke-Collins fueron ampliamente utilizadas en EE.UU., mientras que en Europa se prefería la clasificación de Kiel. Ninguna de ellas fue claramente superior a las otras dada su multiplicidad, la ausencia de una terminología común y la variación en los criterios histológicos. Una iniciativa del "National Cancer Institute" por establecer una terminología común produjo como resultado la clasificación "Working

Formulation" (1982).(5) Esta clasificación, fácilmente aplicable carecía de bases biológicas, ya que, en realidad, había pretendido ser un diccionario entre clasificaciones más que una clasificación en sí misma. No fue hasta los años 90 del siglo XX cuando un grupo de patólogos desarrolló con los conocimientos actualizados la "Revised European-American Lymphoma Classification System" (REAL). Esta clasificación incorporó los datos de inmunohistoquímica y genética, fruto del mayor conocimiento de la célula linfoide, y supuso una unificación de las clasificaciones y la primera aproximación a la definición de las diferentes categorías como verdaderas entidades clínico-biológicas. Aunque varias de las clasificaciones anteriores contemplaban subtipos histológicos de linfomas agresivos con predominio de células grandes, fue la clasificación REAL la que consideró como categoría específica al LDCGB, uniendo en una categoría única formas como el linfoma inmunoblástico previamente separado.(6, 7) En 2001, los conceptos de la REAL fueron asimilados en la clasificación de la OMS.(8) Esta clasificación, fue rápidamente adoptada dada la integración funcional de los conceptos biológicos y clínicos. Recientemente, la clasificación de la OMS se ha actualizado (2008) y se ha centrado en dos aspectos fundamentales: la identificación de los eventos tempranos de la linfomagénesis y los diferentes pasos de la trasformación linfoide neoplásica. Y, en segundo lugar, en la superposición entre diferentes categorías.(9) Por lo que hace al LDCGB, su consideración como una entidad específica se ha mantenido, pero los hallazgos genéticos y moleculares –con sus consecuencias clínicas- han sugerido que estemos ante una entidad heterogénea dentro de la cual se pueden distinguir al menos dos grandes

grupos. La última parte de la introducción está específicamente dedicada al tema de la heterogeneidad del LDCGB y sus consecuencias biológicas y clínicas.(10)

LDCG en los diferentes sistemas clasificatorios de las neoplasias linfoides

- **Rappaport (1966): linfoma histiocítico difuso**
- **Kiel (1974):**
  - **linfoma centroblástico**
  - **linfoma inmunoblástico-B**
  - **linfoma anaplásico de células grandes-B**
- **Luke-Collins (1974):**
  - **linfoma de células centro-foliculares grandes hendidas**
  - **linfoma de células centro-foliculares grandes no hendidas**
  - **linfoma inmunoblástico-B**
- **Working Formulation (1982):**
  - **linfoma difuso mixto de células grandes y pequeñas (grupo F)**
  - **linfoma difuso de células grandes (grupo G)**
  - **linfoma de células grandes inmunoblástico (grupo H)**
- **REAL (1994) y OMS (2001):**
  - **linfoma difuso de células grandes B**
- **OMS (2008):**
  - **linfoma difuso de células grandes B, NOS**

Tabla 1. Evolución del LDCGB en la clasificación de las neoplasias linfoides.

1.2. Anatomía patológica

En la biopsia ganglionar se observa una invasión difusa por células de tamaño grande, núcleo vesicular con nucléolos muy visibles y citoplasma basófilo. Dichas células son morfológicamente parecidas a los centroblastos o a los inmunoblastos que se ven fisiológicamente en un ganglio linfático reactivo. La proliferación es de origen B, siendo el fenotipo más común CD19+, CD20+, CD79a+, CD45+, CD5-/+ y CD10-/+.

Como precisamente la caracterización morfológica, inmunofenotípica y molecular forma parte de los objetivos de la presente tesis doctoral, los aspectos histológicos se detallan en un apartado específico. (Página 29)

1.3. Características clínicas

El LDCGB es una neoplasia heterogénea de curso agresivo, pero potencialmente curable.(11) Afecta predominantemente a varones adultos (ratio varones/mujeres: 1,3/1). Aunque la edad mediana de aparición se sitúa en la séptima década de la vida, el espectro de edad es muy amplio y también se presenta en la infancia y en el adulto joven. La mayoría de los casos son formas “de novo” pero también pueden ser la resultante de la progresión o transformación de una enfermedad linfoproliferativa previa (leucemia linfática crónica, linfoma folicular u otros tipos de linfomas indolentes).(10) Al diagnóstico la mayoría de los pacientes presentan adenopatías rápidamente progresivas, y hasta un 60% de los enfermos pueden presentar síntomas B. Como

todos los linfomas su estadificación se sigue basando en el sistema de "Ann Arbor" desarrollado en los años 70 del siglo XX, aunque su valor pronóstico ha disminuido considerablemente. (Tabla 2) Aproximadamente un 30% de los pacientes se encuentran en estadios precoces de la enfermedad. Si bien es una enfermedad de predominio ganglionar, un 30-40% de los pacientes tienen afectación de sitios extraganglionares (EG) como el tracto gastrointestinal, hueso, piel, tiroides y las gónadas. Entre un 20-30% de los pacientes presentan afectación de la médula ósea. La frecuencia de infiltración del sistema nervioso central al diagnostico no va más allá del 1-2% al diagnóstico, pero puede ser mayor a lo largo de la evolución, de manera que entre un 5-10% de enfermos presentan infiltración del SNC en la recaída o progresión de la enfermedad. Alrededor del 50-60% de los enfermos presentan cifras elevadas de LDH y β2-microglobulina (β2m) séricas, lo que es importante por su valor pronóstico.(10) Con los tratamientos actuales, 70-80% de los pacientes alcanza una respuesta completa. Aunque una proporción de ellos recae posteriormente, se puede decir que 60-70% de los enfermos se curan del LDCGB.

Sistema de Estadificación "Ann Arbor"
❖ Estadio I: afectación de un solo territorio ganglionar o una localización (EG única (IE)
❖ Estadio II: dos o más territorios ganglionares afectos del mismo lado del diafragma, o una afectación EG con un territorio ganglionar regional local (IIE)
❖ Estadio III: afectación ganglionar en ambos lados del diafragma
❖ Estadio IV: Afectación difusa diseminada de uno o más órganos EG (hígado, médula ósea, pulmón, etc.), con o sin afección ganglionar

Tabla 2. Sistema de Estadificación "Ann Arbor"

1.4. LDCGB ganglionares y extraganglionares

Una tercera parte de los LNH, y dentro de éstos aproximadamente la mitad de los LDCGB, tiene un origen primario diferente al territorio ganglionar (LDCGB-G), usualmente denominados linfomas extraganglionares (LDCGB-EG).(12, 13) Durante las últimas dos décadas las incidencia de los linfomas se ha incrementado, en especial los LDCGB-EG. Si bien, este grupo de linfomas no son reconocidos como una variante del LDCGB dentro de la clasificación de la OMS, son muchas las publicaciones que sugieren que la etiopatogenia, las características biológicas y el pronóstico de los LDCGB-EG serían distintos de los ganglionares.(13) Se ha sugerido que pueden existir diferencias genéticas entre los linfomas de origen

primario ganglionar y extraganglionar, dentro de ellas se encuentran alteraciones en los genes c-MYC, BCL-6, REL y FAS (mayor frecuencia en el LDCGB-EG).(14) Desde el punto de vista clínico, los pacientes afectos de LDCGB-EG presentan características favorables (estadio temprano, ausencia de infiltración medular, nivel de LDH normal y un índice pronóstico de riesgo bajo), a diferencia de los LDCGB-G que se presentan con características clínicas desfavorables. En términos de respuesta al tratamiento, riesgo de recaída y supervivencia global (SG), la variante extraganglionar del LDCGB presenta un notable mejor pronóstico que los linfomas de origen primario ganglionar. Todos estos hallazgos hacen pensar que los LDCGB-EG podrían tener un comportamiento clínico-biológico particular, y podrían ser considerados como un subgrupo y/o subtipo específico dentro de los linfomas difusos de célula grande.(15)

1.5. Factores pronósticos en el LDCGB

En la práctica clínica la heterogeneidad del LDCGB es evidente por las diferentes formas de presentación, subgrupos, subtipos y variantes. A pesar del progreso en el conocimiento de la biología del linfocito B y la notable mejoría del pronóstico de los pacientes con LDCGB, una proporción significativa de estos pacientes (20-30%) son refractarios o eventualmente progresaran a la inmunoquimioterapia (IQT) con R-CHOP.(16) En este sentido, la identificación de factores pronósticos, clínicos y/o biológicos, que permitan identificar los pacientes de alto riesgo es una prioridad. Se han descrito diferentes factores pronósticos para la respuesta y supervivencia en el LDCGB. No obstante, el papel

de estos factores en la era de la inmunoquimioterapia está aún por determinar.

1.5.1. Factores pronósticos clínicos

Son numerosísimas las referencias bibliográficas sobre el papel pronóstico de diferentes variables clínicas, tanto referidas a las características del paciente (edad, sexo, co-morbilidades), de la enfermedad, de la masa y extensión tumoral (masa voluminosa, número de territorios extraganglionares afectos, LDH, beta2-microglobulina, etc.), del tratamiento y de la respuesta al mismo. El Índice Pronóstico Internacional (IPI) ha sido la primera herramienta utilizada para predecir la supervivencia en los pacientes con LDCGB. La combinación de cinco parámetros clínicos de fácil medición permite establecer 4 grupos de riesgo (bajo, intermedio-bajo, intermedio-alto y alto) con una SG a los 5 años del diagnóstico que oscilaba en la serie inicial entre 26-73%.(17) (Tabla 3 y Figura 1)

Índice Pronóstico Internacional (IPI)
1. Edad >60 años
2. Estado general mediante la escala "Eastern Cooperative Oncology Group" (ECOG) >1
3. Nivel sérico de LDH elevado
4. Estadio de Ann Arbor avanzado, III-IV
5. Número de localizaciones extraganglionares >1

Tabla 3. Parámetros del Índice Pronóstico Internacional (IPI).(17)

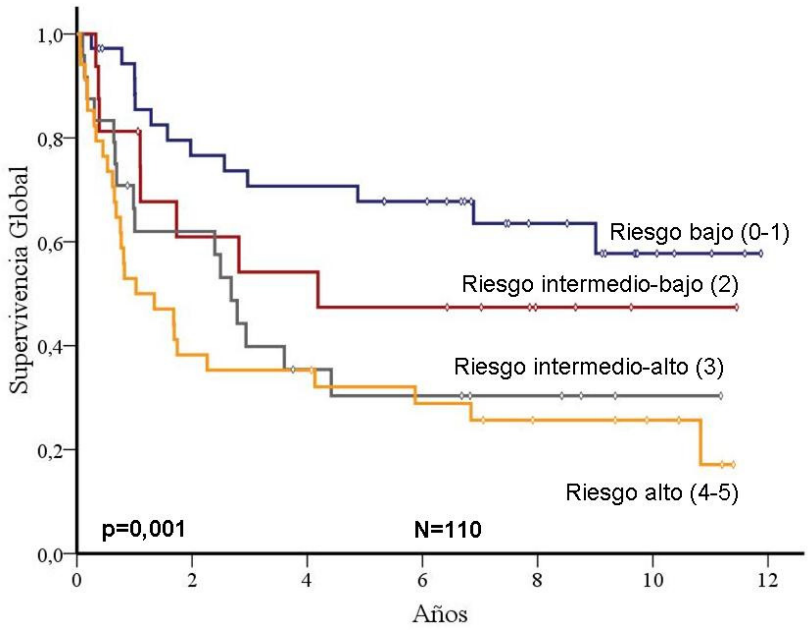

Figura 1. Supervivencia global de 110 pacientes afectos de LDCGB tratados con CHOP, en el Hospital Clínic según el Índice Pronóstico Internacional (IPI).

Sin embargo, el IPI fue elaborado en la era pre-rituximab, y su utilidad en grupos de pacientes tratados con inmunoquimioterapia se ha cuestionado. Uno de los cambios sugeridos ha sido la reorganización de los mismos parámetros del IPI en un índice revisado denominado Índice Pronóstico Revisado (R-IPI) que estratifica en tres los grupos de riesgo: muy favorable (ningún parámetro adverso), favorable (1-2 parámetros adversos) y desfavorable (3-5 parámetros adversos).(18) (Figura 2) En cualquier caso, tanto el IPI como el R-IPI identifican un grupo de pacientes de mal pronóstico (SG-5 años inferior al 50%) en los que es prioritario la identificación de factores biológicos que permitan establecer potenciales dianas terapéuticas.

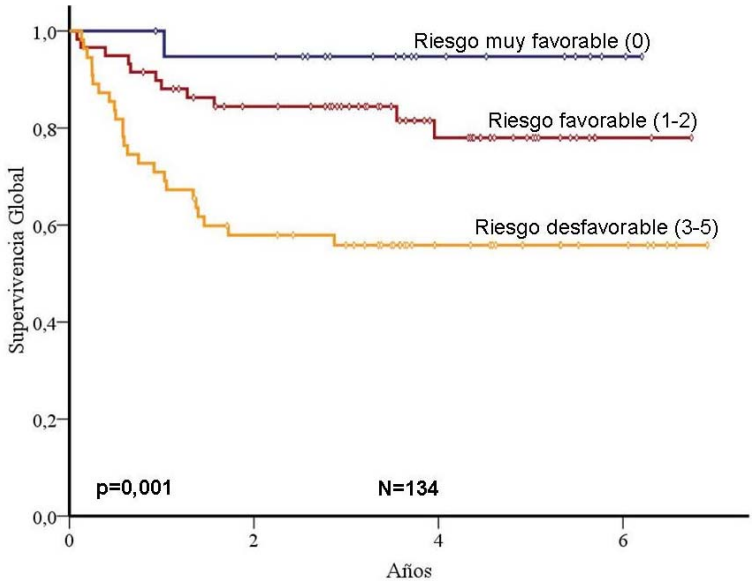

Figura 2. Supervivencia global de 134 pacientes afectos de LDCGB tratados con R-CHOP, en el Hospital Clínic de acuerdo al Índice Pronóstico Revisado (R-IPI).

1.5.2. Factores pronósticos biológicos

En la era pre-rituximab, un número elevado de factores biológicos fueron evaluados y considerados de valor pronósticos en el LDCGB.(19, 20) No obstante, la introducción de la inmunoquimioterapia ha puesto en duda el valor de tales factores.

1.5.2.1. Moléculas reguladoras del ciclo celular

- TP53: es un gen supresor de tumores que actúa como factor de transcripción multifuncional. Interviene en la parada del ciclo celular, la apoptosis, la diferenciación celular, la replicación del DNA y estabilidad genómica.(21, 22) Se detectan mutaciones de TP53 en el 18-30% de los pacientes con LDCGB y están asociadas a un pronóstico desfavorable en algunos estudios(23-25),

pero no en otros.(26-28) La forma mutada de p53 presenta una vida media prolongada a diferencia de la forma germinal, esto le permite ser detectada mediante las técnicas de inmunohistoquímica. Sin embargo, la correlación entre las mutaciones TP53 y la detección inmunohistoquímica de p53 es inexacta.(19)

- Ciclina D: la familia de proteínas ciclina D media la transición de las células de la fase G1 a la fase S, mediante la activación de CDK4 y CDK6.(29) Un reciente estudio ha demostrado que la expresión elevada de ciclinas D2 y D3 están asociadas a un pronóstico adverso en el LDCGB.(30-33)
- Ki-67: es un antígeno nuclear expresado por las células en división. El significado pronóstico de Ki-67 en el LDCGB es controvertido, por un lado debido a la falta de consenso en la estandarización del punto de corte.(34-37) Además, no se ha observado relación entre el porcentaje de Ki-67 y la sensibilidad genética de las células tumorales a la quimioterapia.(19)

1.5.2.2. Proteínas apoptóticas

- BCL2: es una proteína anti-apoptótica localizada principalmente en la membrana interna de la mitocondria.(38) La sobre-expresión de esta proteína en el LDCGB se observa entre un 47-58%, y está relacionada con la con la inhibición de la apoptosis. Diferentes estudios realizados series de pacientes tratados con QT demostraron el impacto desfavorable en el pronóstico de

los pacientes con sobre-expresión de BCL2.(26, 28, 36) Sin embargo, en la era de la IQT su valor pronóstico ha sido puesto en duda.(39) Probablemente, una de los problemas relacionados radica en la dificultad para encontrar un punto de corte óptimo que relacione la sobre-expresión con el pronóstico de los pacientes.(19)

1.5.2.3. Proceso de diferenciación linfocitaria

- BCL6: es un marcador de maduración centrogerminal.(40, 41) En la era pre-rituximab, la sobre-expresión de BCL6 estaba relacionada con un pronóstico favorable del LDCGB. Sin embargo, la adición de rituximab ha beneficiado en especial al grupo de pacientes BCL6 (+), por ello su valor pronóstico ha sido puesto en duda.(42)
- FOXP1: es un factor de transcripción expresado en las células linfoides B.(43) La sobre-expresión de FOXP1 está relacionada con un pronóstico adverso.(44, 45) Sin embargo, la clonación del anticuerpo y la falta de estandarización del punto de corte dificultan su reproducibilidad.(19)
- LMO2: es un factor de transcripción relacionado con el centrogerminal. Su sobre-expresión está relacionada con un pronóstico favorable.(46, 47)

1.5.2.4. Subgrupos moleculares

En la era de la inmunoquimioterapia, se ha demostrado el impacto pronóstico del origen del LDCGB. El subgrupo molecular denominado activado (ABC) presenta un pronóstico

adverso, en comparación con el subgrupo centrogerminal B (CGB) en pacientes tratados con y sin rituximab. En la actualidad, es el factor pronóstico biológico con mayor impacto en la supervivencia. El valor pronóstico de los subgrupos moleculares, ha sido reproducido en diferentes series de pacientes en la era de la inmunoquimioterapia.(47-49) (Figura 3).

Los intentos por validar los diferentes algoritmos inmunohistoquímicos (Colomo, Hans y Muris) en la era pre-rituximab, en series de pacientes tratados con IQT son objeto de debate. Durante la era de la inmunoquimioterapia se han elaborado dos algoritmos más (Choi y Tally) con impacto en el pronóstico (teniendo el subtipo fenotípico CGB un mejor pronóstico que el fenotipo no-CGB). Sin embargo, está información no se ha podido validar en series independientes.(37) Por ello, el valor pronóstico de los algoritmos inmunohistoquímicos es desconocido. Estos aspectos, relacionados directamente con los objetivos de la presente tesis doctoral, se detallan en el apartado de caracterización del LDCGB (página 38).

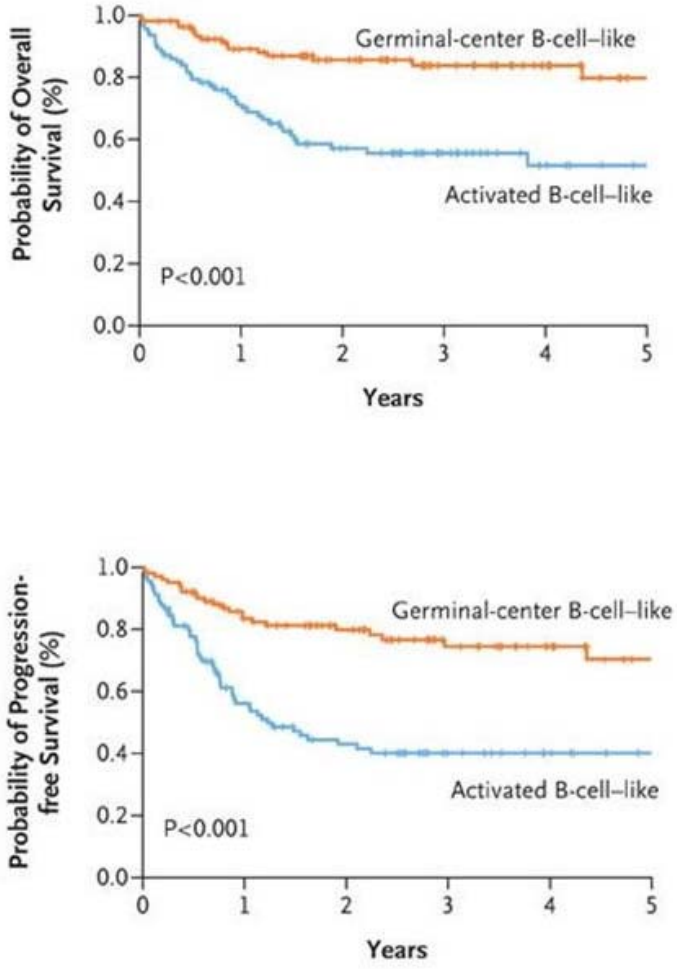

Figura 3. Supervivencia global y supervivencia libre de progresión de acuerdo al origen del linfoma (CGB frente a ABC), en una serie de pacientes tratados con R-CHOP.(49)

1.6. Tratamiento del LDCGB

La evolución en el tratamiento del LDCGB representa uno de los progresos más importantes en el campo de las neoplasias. Dos han sido los pasos cruciales en el último medio siglo: la poliquimioterapia que se empezó a usar en los años 70 del siglo XX y la inmunoterapia (e inmunoquimioterapia) utilizada masivamente desde el año 2000. Esto ha convertido una enfermedad antes fatal en la mayoría de los pacientes en una enfermedad curable en una alta proporción de casos.(50) Diferentes estrategias terapéuticas han sido utilizadas durante las últimas tres décadas, entre las que podemos destacar la poliquimioterapia y la inmunoquimioterapia, la intensificación mediante el trasplante autólogo, los inhibidores de proteosoma y los inmunomoduladores.

1.6.1 Regímenes de poliquimioterapia

El uso de esquemas de quimioterapia (QT) basados en antraciclínicos, llevó a considerar al LDCGB en una enfermedad con potencial curativo.(51) El régimen quimioterápico denominado CHOP (ciclofosfamida, doxorrubicina, vincristina y prednisona) se convirtió durante 20 años en el tratamiento estándar de los pacientes con LDCGB.(51) Este régimen, permite alcanzar una tasa de remisión completa (RC) entre 40-45% de los pacientes con un impacto favorable en la SG (40-50). Los denominados “regímenes de tercera generación” (MACOP-B, ProMACE-CytaBOM, mBACOD) que incluyen en sus combinaciones dosis bajas de metotrexato, bleomicina, etopósido

y citarabina, fueron prometedores en sus resultados iniciales incrementando la tasa RC. Sin embargo, estos regímenes más complejos en su administración y con mayor toxicidad no presentaron mejores tasas de respuesta ni de supervivencia que el CHOP convencional en estudios aleatorizados en fase III.(52)

Más recientemente, como método para mejorar los resultados, se ha sugerido el aumento de la densidad de tratamiento, lo que consiste en acortar el intervalo entre dosis. La forma más popular es convertir el CHOP-21 convencional, esto es, administrado cada 21 días en el CHOP-14, administrado cada 2 semanas.

1.6.2. Inmunoquimioterapia con rituximab

La introducción de rituximab (un anticuerpo monoclonal quimérico humano/murino dirigido contra el antígeno CD20 expresado en la superficie de los linfocitos B) en el tratamiento de los linfomas, en combinación con el esquema de quimioterapia CHOP inició la denominada era de la IQT. En 2002, el "Groupe d'Etude des Lymphomes de l'Adulte" (GELA) publicó los primeros resultados del estudio LNH98-5. Este estudio comparó CHOP frente a R-CHOP en pacientes mayores (60-80 años) con LDCGB, y demostró un incremento significativo de la tasa de RC y una mejoría en la supervivencia libre de evento (SLE) y la SG.(50) (Figura 4) La adición de rituximab incrementó de forma significativa la tasa de RC (76% frente a 63%) cuando se comparó R-CHOP frente a CHOP. Subsecuentemente, tres ensayos clínicos han confirmado el beneficio de la IQT en varios grupos de pacientes con LDCGB, y han convertido hoy por hoy a R-CHOP en el tratamiento estándar. Los ensayos clínicos "US Intergroup" y el "RICOVER-60" evaluaron la IQT en pacientes

mayores con LDCGB,(53, 54) mientras que el "MabThera International Trial" (MInT) investigó su uso en pacientes jóvenes (edad ≤ 60 años).(55) Los resultados del GELA son en la actualidad los más maduros, con una mediana de seguimiento de 10 años, una tercera parte de los pacientes que recibieron tratamiento con R-CHOP (36,5%) se encuentran libre de evento, respecto a 20% en el brazo con CHOP. La SG a 10 años fue 43,5% frente a 27,6% (R-CHOP frente a CHOP, respectivamente). Estos datos, indican que la adición de rituximab mejora la SLP y la SG, con un incremento global del 16% a favor del R-CHOP. Actualmente, se desconoce si la adición de rituximab a los regímenes intensivos de quimioterapia puede aportar beneficios. Diferentes estudios que comparan R-CHOP21 con R-CHOP14 se encuentran en marcha.(56)

En conjunto, todos los datos anteriores han hecho de la inmunoquimioterapia (y del R-CHOP como su paradigma) el tratamiento estándar para cualquier paciente con LDCGB.

Hay que indicar en cualquier caso que la efectividad de la inmunoquimioterapia ha sido cuestionada en algunos casos, por ejemplo en pacientes BCL2+, BCL6- o aquellos con un fenotipo no-CGB.(42, 47, 48, 57) Además, existe poca información sobre la eficacia de la IQT en pacientes con LDCGB-EG.

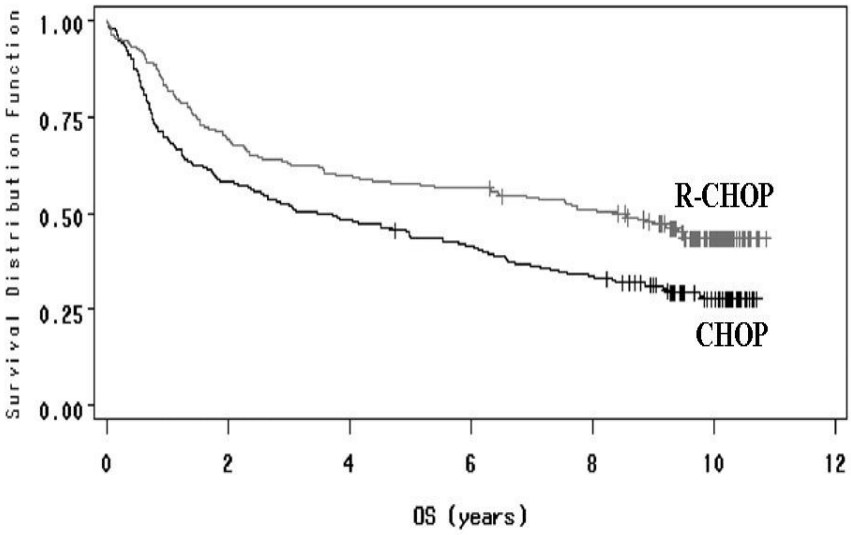

Figura 4. Supervivencia global en pacientes tratados con CHOP y R-CHOP. La supervivencia mediana fue 3,5 años (IC 95%: 2,2-5,5) frente a 8,4 años (IC 95%: 5,4-no alcanzada) para lo pacientes que recibieron CHOP frente a R-CHOP, respectivamente; p<0,0001.(50)

1.6.3. Trasplante autólogo de progenitores hematopoyéticos (TASP)

La recaída en el LNH no sólo es causada por mecanismos intrínsecos de resistencia tumoral (resistencia genética) sino por una disminución en la sensibilidad de las células tumorales a la quimioterapia (resistencia cinética).(58, 59) Partiendo de esta hipótesis, diferentes estrategias terapéuticas han sido diseñadas. Una de ellas, consiste en realizar una intensificación con diferentes agentes quimioterápicos de acción no cruzada después del tratamiento de inducción. El desarrollo de estrategias basadas en altas dosis de quimioterapia, seguido de un TASP demostró un significativo impacto en la SG de los pacientes en recaída y/o progresión tras un tratamiento de primera línea.(59, 60) Sin

embargo, el beneficio de la intensificación con TASP como tratamiento de primera línea es controvertido.(61) Si bien, de manera global se observó un incremento en la tasa de RC y una mejoría significativa en la supervivencia libre de progresión (SLP), no se encontró impacto en la SG. (Figura 5) Más importante aún, fue el impacto negativo en la SG, observado en los pacientes de riesgo bajo cuando se comparó frente a la quimioterapia convencional. Aunque, en la actualidad la intensificación mediante un TASP como tratamiento de primera línea se utiliza en los pacientes de riesgo alto, no hay un probado beneficio que justifique su uso por fuera de ensayos clínicos.(59)

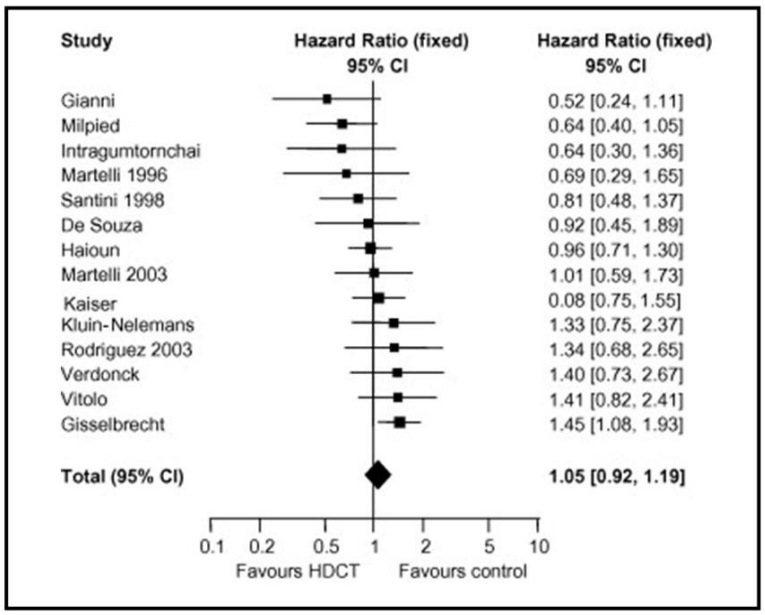

Figura 5. Resultados del más importante metanálisis realizado para SG en pacientes con LNH que recibieron QT convencional frente a dosis altas de QT seguido de un TASP. Cada cuadro índica la razón de riesgo de cada estudio. El tamaño de cada cuadro es proporcional al tamaño de la muestra y el número de eventos. Las líneas horizontales representan el intervalo de confianza (IC) del 95% en cada estudio. El rombo, representa el IC para el conjunto de razón de riesgo. El valor negativo índica una tasa reducida en la razón de riesgo para la SG a favor del TASP.(62)

1.6.4. Inhibidores de proteasoma

A partir de los estudios de expresión génica se ha establecido que los subgrupos moleculares del LDCGB (CGB y ABC) son entidades patogénicas diferentes. En este sentido, en el subgrupo de LDCGB ABC se evidenció sobre-expresión del factor nuclear kB (NF-kB). La expresión constitutiva de esta vía de señalización bloquea la respuesta apoptótica de los antraciclínicos y podría ser responsable en parte del pronóstico desfavorable en este subtipo particular de LDCGB. (63, 64) La introducción de la IQT, que ha sido el mayor logro en el tratamiento del LDCGB durante la última década, tampoco ha demostrado incrementar significativamente el pronóstico en el LDCGB-ABC.(49) El inhibidor de proteasoma denominado bortezomib, conocido por su actividad contra el mieloma múltiple, inhibe la actividad de (NF-kB) a través del bloqueo en la degradación de la quinasa IkB.(63, 65, 66) Los resultados iniciales de un estudio conducido por Dunleavy et al, sugierieron que el bortezomib podría incrementar el efecto de la QT en el subgrupo de LDCG-ABC. Ruan et al., recientemente han publicado los resultados de un ensayo clínico, que evaluó dosis escaladas de bortezomib más R-CHOP frente a R-CHOP según el fenotipo del LDCG (CGB frente a no-CGB) encontrando beneficio en terminos de supervivencia a favor en los pacientes con LDCGB de fenotipo no-CGB en el grupo que recibió bortezomib. Sin embargo, la toxicidad neurológica en forma de neuropatía periférica fue elevada (49%).(51)

1.6.5. Inmunomoduladores

Los nuevos derivados de la talidomida (IMiDs) como la lenalidomida, ejercen su actividad a través de la activación del sistema inmune, modifican la respuesta mediada por citoquinas, e inhiben la angiogénesis.(67) Estos agentes han demostrado acción sinérgica en combinación con rituximab.(68) En especial, se ha observado beneficio de los IMiDs en el grupo de linfomas de origen ABC. La eficacia y seguridad de lenalidomida en pacientes con LNH agresivos en recaída o refractarios al tratamiento ha sido evaluada en diferentes ensayos clínicos con resultados aceptables y adecuado perfil de seguridad.(67) En la actualidad, están en marcha ensayos clínicos que evalúan su utilidad en el tratamiento rescate en combinación con quimioterapia y en el mantenimiento de los pacientes con LDCGB.

2. Caracterización del LDCGB. Importancia de la clasificación molecular

El LDCGB aunque considerado como una categoría en la actual clasificación de la OMS es una neoplasia heterogénea de difícil caracterización mediante técnicas convencionales. Las diferentes herramientas que disponemos en la actualidad: morfología, inmunofenotipo, genética, biología molecular y el abordaje clínico permiten en el LDCGB definir variantes, subgrupos, y subtipos/entidades.

2.1. Morfología

Constituido por células linfoides de un tamaño grande y núcleo pleomórfico, el LDCGB presenta un patrón de crecimiento difuso que altera parcial o totalmente la arquitectura ganglionar. La afectación ganglionar parcial suele ser interfolicular y en un menor porcentaje sinusoidal. El tejido perigangliónar de manera frecuente se encuentra afectado, y se puede observar la presencia de bandas fibroescleróticas. De acuerdo al tipo celular presenta tres variantes morfológicas:

a. Centroblástica: se caracteriza por la presencia de centroblastos, que son células linfoides de tamaño mediano/grande de núcleo vesicular con la cromatina fina y la presencia de 2-3 nucleolos. Es la variante más frecuente y en la mayoría de casos la población es

polimorfa (centroblastos e inmunoblastos). En algunos casos los centroblastos pueden presentar el núcleo multilobulado, este hallazgo se encuentra asociado a formas extraganglionares del linfoma. (Figura 6).

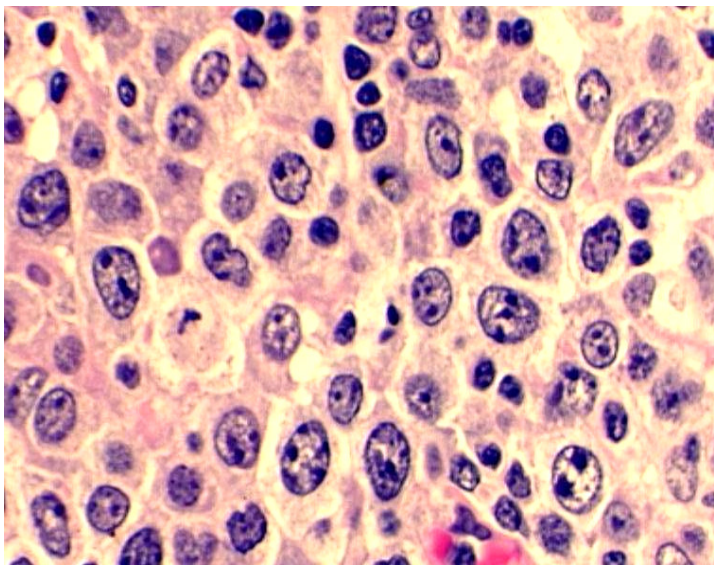

Figura 6. LDCGB variante centroblástica
Hematoxilina & eosina 100X

b. Inmunoblástica: en esta variante la población tumoral se encuentra constituida en más del 90% por inmunoblastos, que son células de un tamaño grande, citoplasma intensamente basófilo con núcleo redondo que presenta un único nucleolo de localización central. (Figura 7) Esta variante puede ser difícil de diferenciar de una afectación extramedular por un linfoma plasmablástico. El impacto pronóstico desfavorable de esta variante ha sido debatido durante los últimos 15 años y se mantiene en la actualidad.(37)

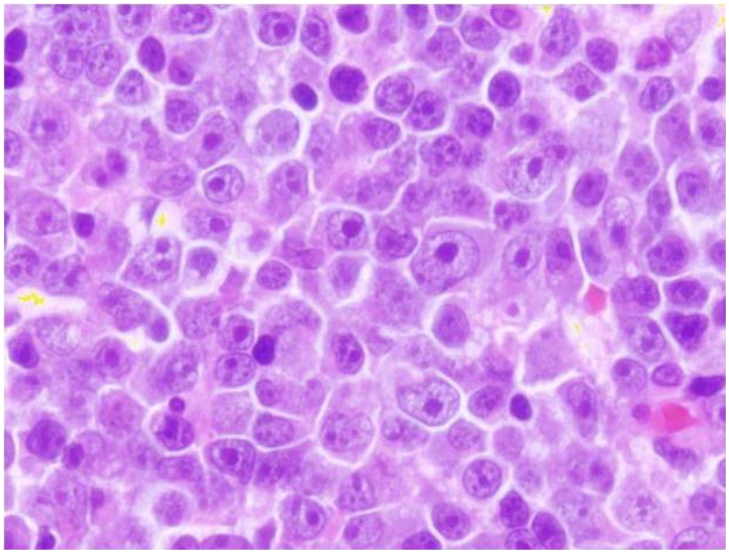

Figura 7. LDCGB variante inmunoblástica
Hematoxilina & eosina 100X

c. Anaplásica: en este caso las células son pleomórficas, de aspecto poligonal o bizarro. En muchas ocasiones el tipo celular recuerda a las células de Reed-Sternberg, características del linfoma Hodgkin. Estas células presentan un patrón de crecimiento sinusoidal y/o cohesivo que puede semejar a un carcinoma indiferenciado. (Figura 8). Esta variante es biológica y clínicamente diferente del linfoma de células grandes anaplásico que deriva de linfocitos T citotóxicos.

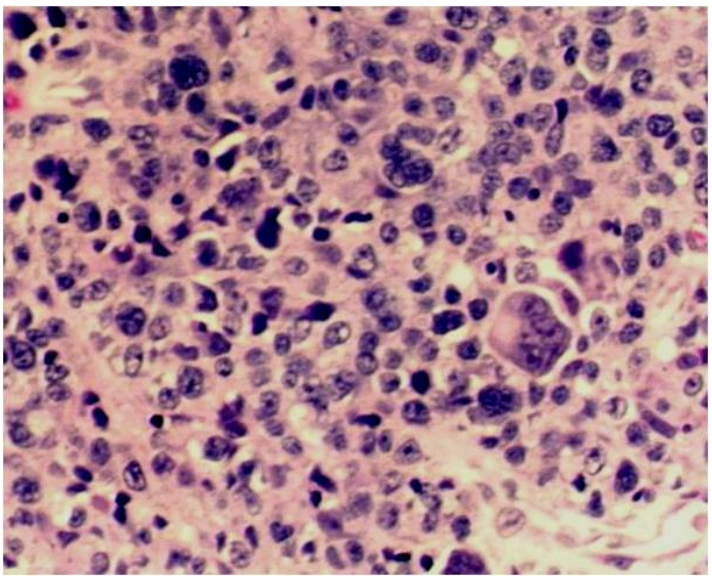

Figura 8. LDCGB variante Anaplásica
Hematoxilina & eosina 100X

Si bien la morfología permite de cierta manera categorizar el LDCGB, su baja reproducibilidad intra e interobservador es la principal desventaja.(1, 7) Por otro lado, el valor pronóstico de las diferentes variantes morfológicas es controvertido, en especial el pronóstico desfavorable observado en la variante inmunoblástica.(37, 69, 70)

2.2. Estudio inmunofenotípico

Las células neoplásicas expresan antígenos de línea B como CD19, CD20, CD22 y CD79a. En el 50-75% de los casos las células expresan inmunoglobulinas (Igs) en su superficie o en el citoplasma.(10) La expresión de antígenos de diferenciación plasmocítica como CD38 y CD138 (1328) es poco frecuente, y no se correlaciona con la expresión de Igs.(10) En la variante anaplásica, se puede observar la expresión de CD30. El índice proliferativo mediante el antígeno Ki-67 suele estar elevado (>40%) y en algunos casos es superior al 90%. Los casos de LDCGB con co-expresión de CD5 presentan una frecuencia del 10%, suelen corresponder a eventos de nueva aparición, y en pocas ocasiones puede estar asociado a una trasformación histológica de la LLC. El LDCGB-CD5+, está considerado como un subgrupo inmunofenotípico específico en la actual clasificación de la OMS y puede ser diferenciado de la variante blastoide del linfoma de células del manto por la ausencia de expresión de ciclina D1.(10) Desde el punto de vista clínico, el LDCGB-CD5+ tiene una edad mediana de aparición que está sobre los 65 años, predomina en el sexo femenino, suele diagnosticarse en estadios avanzados de la enfermedad y es frecuente la afección extraganglionar. Todo ello, le confiere un pronóstico desfavorable, con una SG-5 años inferior al 50%.(71, 72)

2.3. Perfiles de expresión génica (GEP)

El conocimiento de la linfomagénesis y el desarrollo de las técnicas de biología molecular ha permitido identificar, mutaciones hipersomáticas de las inmunoglobulinas en las células linfoides del LDCGB. Este mecanismo está involucrado en la diversificación del repertorio de inmunoglobulinas y ocurre únicamente dentro del centrogerminal de los órganos linfoides secundarios. Este hallazgo sugiere que la célula de origen en el LDCGB puede corresponder a linfocitos del CGB, o en estadios de diferenciación posterior [post-centrogerminal (post-CGB)].(73) Figura 9.

Los perfiles de expresión génica de forma general, cuantifican en paralelo la expresión de cientos o miles de genes. En el caso concreto del LDCGB, Alizadeh et al., en una primera fase utilizaron una plataforma que incluyó la expresión de 17593 genes agrupados de acuerdo a las siguientes firmas:

- Firma genética de células linfoides B "naive", y sometidas a procesos de activación en la sangre periférica
- Centrogerminal
- Celularidad linfoide T
- La proliferación
- Ganglio linfático

Esta plataforma denominada "Lymphochip" fue aplicada sobre muestras de ganglio linfático normal, LDCGB, LLC, LF y linfocitos de la sangre periférica sometidos a procesos de mitogénesis y activación. El resultado de expresión génica de

acuerdo a cada firma se puede observar en la Figura 10. En las muestras correspondientes al LDCGB, se identificaron dos firmas particulares. La primera presentaba sobre-expresión de los mismos genes observados en el grupo CGB. Los genes que destacaban en esta firma fueron: CD10, CD38, el factor nuclear A-myb, OGG1, BCL6, BCL7A, LMO2 y JAW1. Este subgrupo adoptó el nombre de CGB (LDCGB-CGB). (Figura 11) La segunda firma, presentaba sobre-expresión del perfil correspondiente a las células linfoides de la sangre periférica sometidas a procesos de mitogénesis y activación. En este caso, los principales genes sobre-expresados fueron: MUM1/IRF4, FLIP, FOXP1 y BCL2. Este subgrupo adoptó el nombre de ABC (LDCGB-ABC).(74) (Figura 11)

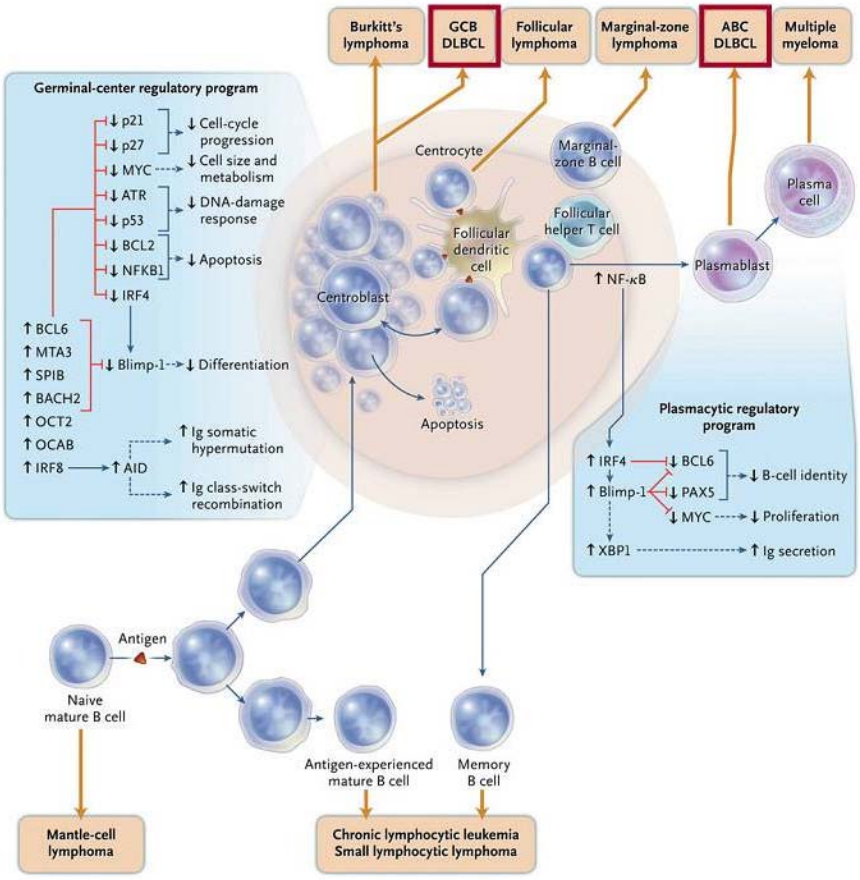

Figura 9. Proceso de diferenciación de la célula linfoide B y la linfomagénesis. Los linfomas pueden originarse en diferentes estadios de desarrollo de la célula linfoide B normal. Una vez se ha realizado la estimulación antigénica el linfocito B, a nivel del CGB, y por intermedio de la deaminasa citidina inductora de activación (AID) sufrirá el proceso de hipermutación de inmunoglobulinas y el "class switching" de la cadena pesada. En este punto intervienen varios factores de trascripción que se encargan de mantener la función e integridad del CGB: BCL6, MTA3, SPIB, BACH2, OCT2, OCAB y IRF8. Este proceso llevará a la conversión de centroblastos a centrocitos. El factor de trascripción MUM1/IRF4 iniciará el proceso de diferenciación plasmocítica. La célula origen del LDCGB podría originarse del CGB o en una fase posterior en la diferenciación plasmocítica. Modificado de Lenz et al..(73)

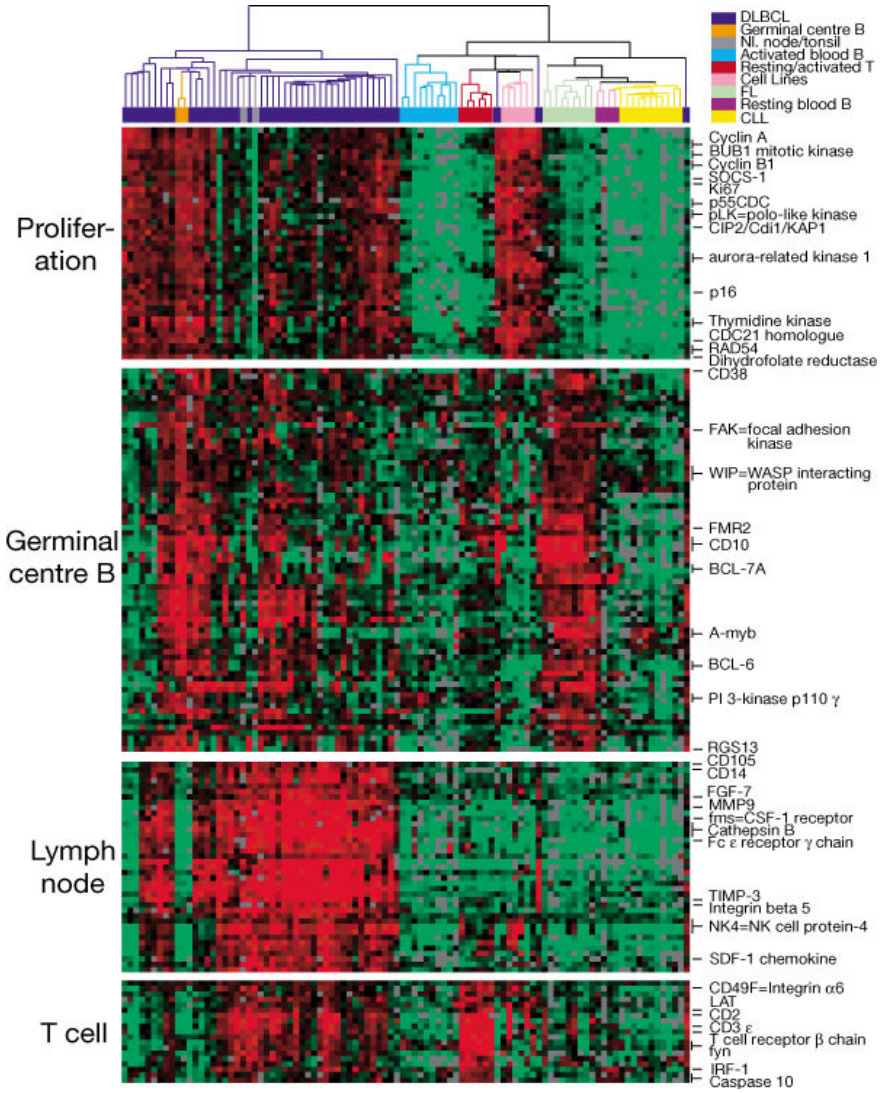

Figura 10. Distribución jerárquica de los perfiles de expresión génica en el LDCGB. Vista ampliada de la expresión de los diferentes genes de acuerdo a grupos comunes: proliferación, centro germinal, ganglio linfático y célula linfoide T.(74)

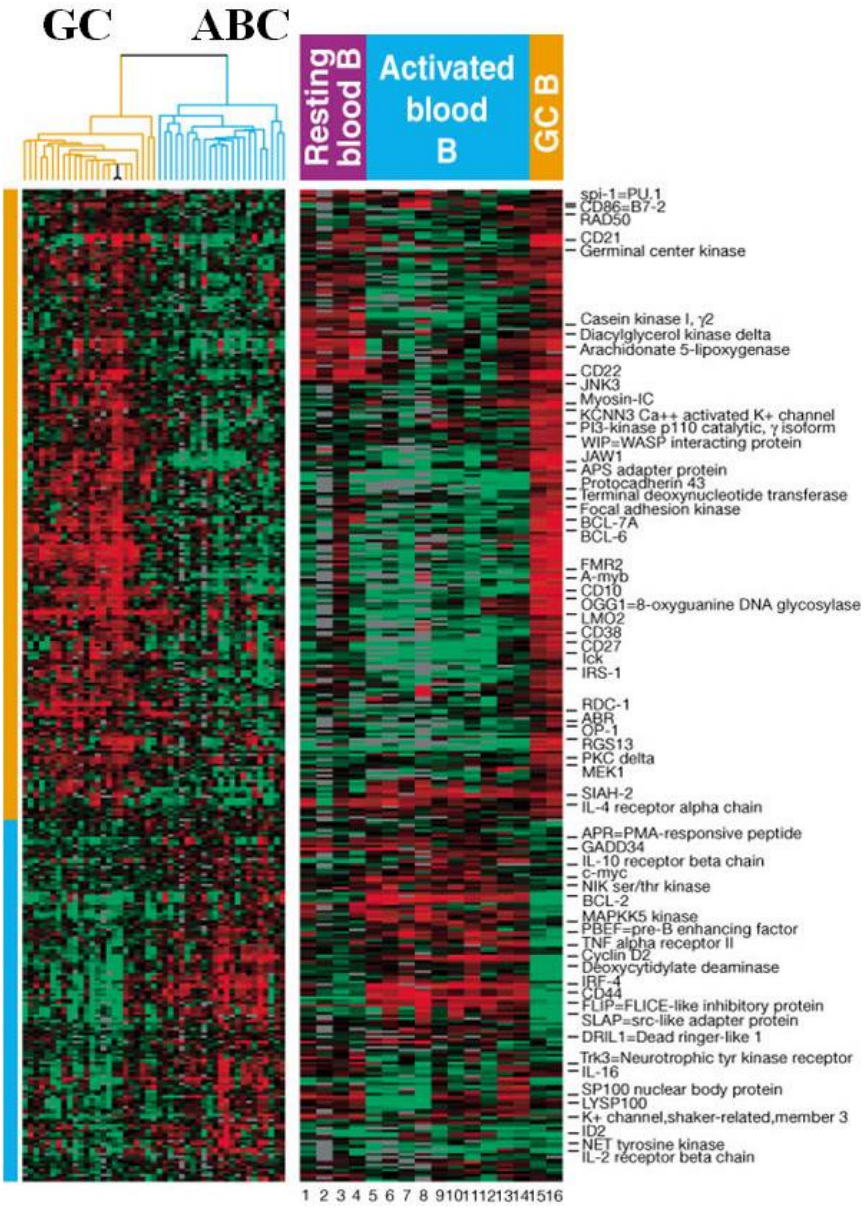

Figura 11. Caracterización del LDCGB de acuerdo al perfil de expresión génica. En el panel de la izquierda se observa la expresión génica de los casos de LCDG en 2 grupos claramente separados. En el panel de la derecha observa la expresión de genes relacionados con los linfocitos B activados y genes relacionados con el CGB. La imagen en conjunto muestra la correlación entre los dos paneles y la caracterización del LDCGB en dos subgrupos CGB y ABC.(74)

Además del sentido puramente biológico de ambos grupos, la caracterización del LDCGB mediante GEP tiene un importante impacto pronóstico, de manera que el grupo CGB tiene una SG más prolongada que el grupo ABC (SG 5-años: 69% frente a 30%, respectivamente; p<0,001).(75) (Figura 12)

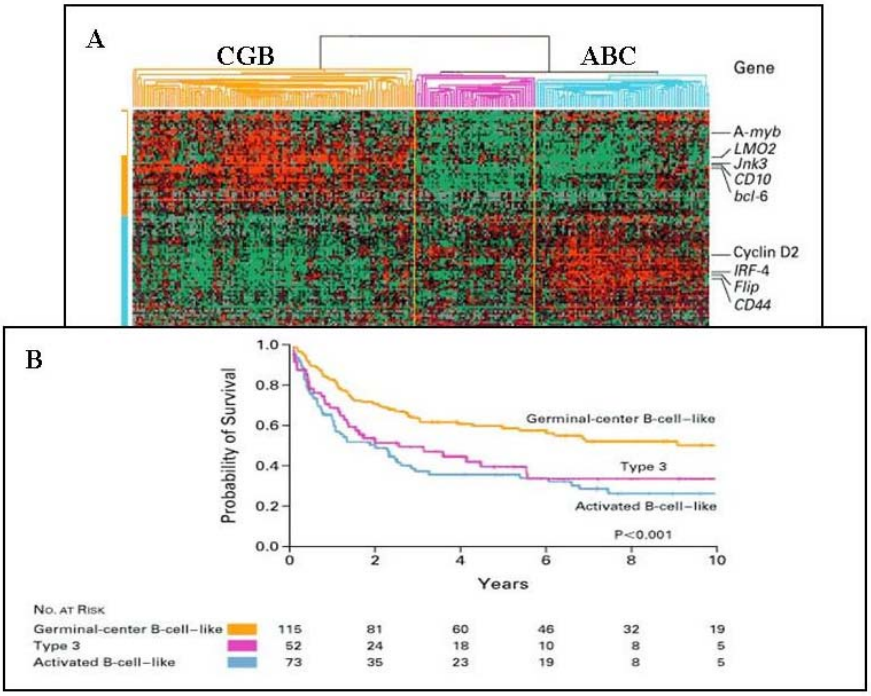

Figura 12. Caracterización del LDCGB de acuerdo al perfil de expresión génica y supervivencia. En la parte superior denominada A, se observa los GEP de 240 pacientes afectos de LDCGB según los diferentes subgrupos (CGB y ABC). En la parte inferior de la figura denominada B, se observa una curva de Kaplan-Meier que estima la supervivencia global de los 240 pacientes según el perfil de expresión génica (CGB frente a ABC).(74)

Los subgrupos moleculares de LDCGB presentan grandes diferencias en su programa celular. Por un lado, el LDCGB-CGB presenta una hipermutación somática de las inmunoglobulinas que le confiere un programa característicamente de centrogerminal. Por el contrario, el LDCGB-ABC tienen un programa de diferenciación plasmocítica con expresión del factor de trascripción XBP1, activación constitutiva de la vía NF-kB y expresión de MUM1/IRF4.(74)

Los estudios de expresión génica, han permitido además identificar el valor del microambiente en el LDCGB. Dos perfiles de expresión denominados, estroma-1 y estroma-2 reflejan la importancia de la celularidad no maligna en el LDCGB. El perfil estroma-1, incluye genes relacionados con el tejido mesenquimal y la matriz extracelular, involucrados en la reacción fibrótica que acompaña al tumor: MMP9, SPARC y CTGF. El perfil estroma-2, está relacionado con genes involucrados en la angiogénesis: VEGF, EGFL7, MMRN2, GPR116 y SPARCL1. Además involucra los genes que codifican par CD31 y el factor Von Willebrand.(49)

2.4. Alteraciones cromosómicas específicas en los subgrupos moleculares del LDCGB

Los estudios de citogenética, han demostrado que el LDCGB presenta diferentes alteraciones que pueden clasificadas según su frecuencia en los subgrupos moleculares:

2.4.1. Alteraciones genéticas en el LDCGB-CGB:

a. Ganancias del cromosoma 12q12.
b. Ganancias del cromosoma 9p21
c. Ganancias del cromosoma 2p12-p16

2.4.2. Alteraciones genéticas en el LDCGB-ABC:

a. Cromosoma 3: ganancia del brazo largo (3q) y trisomía del cromosoma 3. Característicamente estos eventos no se encuentran en el LDCGB-CGB. Las alteraciones que involucran 3q27 resultan en la expresión constitutiva del gen BCL6 que producirá un arresto en la maduración plasmocítica, favoreciendo la proliferación a través de la inhibición de vía apoptótica p53.(76, 77)
b. Cromosoma 18: ganancias y amplificaciones de 18q21-22 que involucran al gen BCL2. Si bien, BCL2 suele estar sobre-expresado con mayor frecuencia en los LDCGB-ABC en pocos casos se encuentra la presencia de la t(14;18). Esto ocurre de forma diferente en los casos de LDCGB-CGB con sobre-expresión de BCL2, en los que siempre se encuentra la t(14;18). Estos datos sugieren que la amplificación de la región 18q21 ocurre preferencialmente en los linfomas que tienen la capacidad para transcribir el gen BCL2. La sobre-expresión de BCL2 conlleva una inhibición de la apoptosis y confiere un pronóstico desfavorable.(77) (14)

Además, las técnicas de PCR cuantitativa a tiempo real (RT-PCR) han mostrado en los dos subgrupos moleculares del LDCGB (CGB y ABC) sobre-expresión en los genes VRK2, XP01, SLC12A, ACTR2, MADH2, MADH4, LOC51320 y PMA1P1. Por el contrario, en los LDCGB-ABC se encuentra sobre-expresión de MIZ1, ME2, MALT1, BCL2 y FVT1. El LDCGB-CGB, presenta sobre-expresión en los genes REL, ASHA2, MDH1 y UGP2. Otra alteración genética que confiere mal pronóstico es el reordenamiento de cMYC, observado en el 15% de los LDCGB. Este reordenamiento, se produce en la mayoría de los casos como consecuencia de la t(8;14) que involucra cMYC en 8q24 (78).

2.5. Estudio de los grupos moleculares mediante inmunohistoquímica

La dificultad en aplicar los GEP en la práctica clínica habitual, debido a su elevado costo y elaborada complejidad, ha llevado a remedar esta información mediante la inmunohistoquímica. En la actualidad, disponemos de una serie marcadores dirigidos contra antígenos de diferenciación CGB y post-CGB y/o no-CGB. Dentro de los marcadores de fenotipo CGB destacan CD10, BCL6, GCET1/Serpina, GCET2/HGAL, LMO2, JAW1 e IRF8. En lo que refiere a marcadores de fenotipo no-CGB sobresalen MUM1/IRF4, FOXP1, XBP1 y BLIMP1. En todos ellos, la expresión en el LDCGB es variable y oscila entre el 40-80%.(10) Con base en la expresión individual de estos marcadores se han construido diferentes algoritmos con el fin de establecer el

fenotipo del linfoma (CGB y no-CGB). Tres de algoritmos fueron construidos en la era pre-inmunoquimioterapia:

a. Algoritmo de Colomo: fue el primer algoritmo elaborado. Por medio de la expresión de MUM1/IRF4, CD10 y BCL6, fue capaz de discriminar tres categorías en el LDCGB: CG-CD10 positivo, CG-CD10 negativo y no-CG. (Figura 13) Sin embargo, este algoritmo no tuvo impacto en el pronóstico.(36)

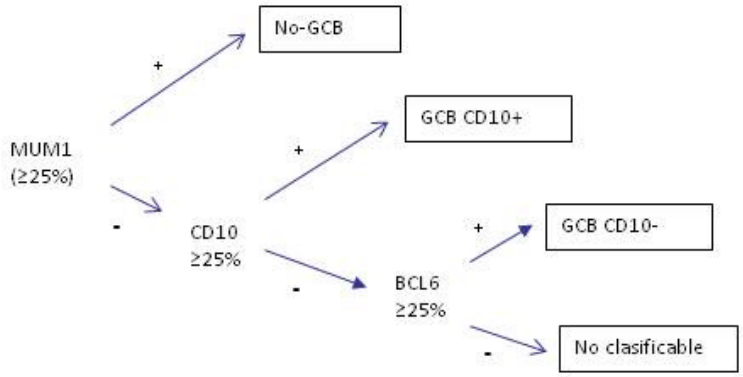

Figura 13. Algoritmo inmunohistoquímico de Colomo.(36)

b. Algoritmo de Hans: utiliza la expresión de CD10, BCL6 y MUM1. Este algoritmo es probablemente el más popular, además de ser el primero que se basó en la correlación con los GEP. El algoritmo de Hans fue capaz de identificar los subgrupos CGB y no-CGB con diferencias significativas en el pronóstico. (Figura 14) Sin embargo, su reproducibilidad en la era post-rituximab ha sido cuestionada.(79)

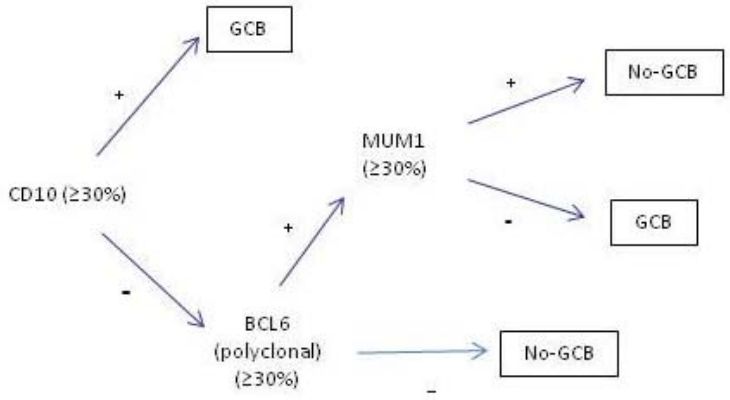

Figura 14. Algoritmo inmunohistoquímico de Hans.(79)

c. Algoritmo de Muris: desarrollado en un grupo de pacientes tratados sin rituximab, este algoritmo en un primer paso a partir de la expresión de CD10 y MUM1/IRF4 (excluyendo BCL6 del algoritmo) permite diferenciar dos categorías fenotípicas (CGB y no-CGB). En un segundo paso, incorpora el valor pronóstico de BCL2 y establece dos grupos pronósticos (grupo1 y 2).(80) (Figura 15)

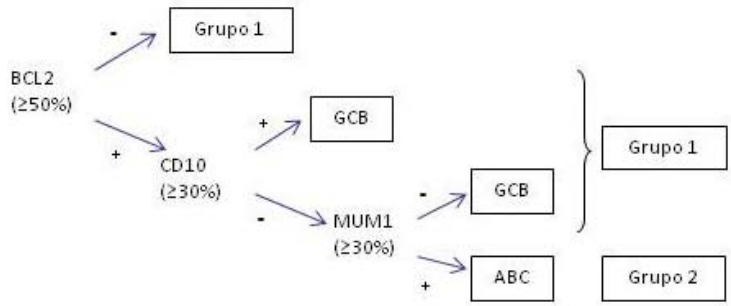

Figura 15. Algoritmo inmunohistoquímico de Muris.(80)

Otros dos algoritmos han sido elaborados en la era de la inmunoquimioterapia:

a. Algoritmo de Choi: se basa en la expresión de GCET1, CD10, BCL6, MUM1/IRF4 y FOXP1.(Figura 16) Este algoritmo fue comparado con los perfiles de expresión génica, con una elevada concordancia.(48)

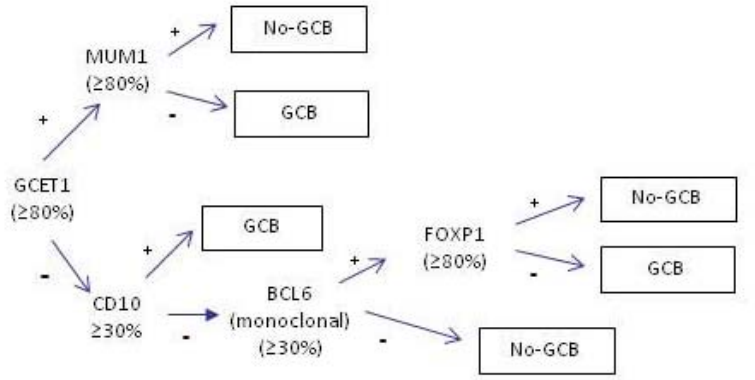

Figura 16. Algoritmo inmunohistoquímico de Choi.(48)

b. Algoritmo "Tally": este algoritmo recientemente publicado utiliza la expresión apareada de antígenos de diferenciación CGB (CD10 y GCET1) y no-CGB (MUM1/IRF4 y FOXP1). Además, incorpora la expresión del marcador de centrogerminal LMO2 para discriminar el fenotipo del linfoma (CGB y no-CGB).(47) (Figura 17).

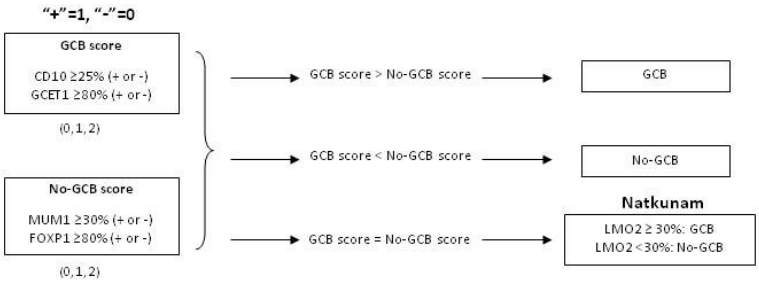

Figura 17. Algoritmo inmunohistoquímico "Tally".(47)

Linfoma difuso de célula grande B, no especifícado (*NOS*)

Variantes morfológicas comunes

- Centroblástico

- Immunoblástico

- Anaplásico

❖ ***Subgrupos moleculares***

- Centrogerminal (*CGB*)

- Activado (*ABC*)

❖ ***Subgrupos inmunohistoquímicos***

- LDCG-B CD5-positivo

- Centrogerminal (*GCB*)

- No centrogerminal (*no-GCB*)

Tabla 4. Subtipos y entidades del LDCGB de acuerdo a la morfología, biología molecular e inmunofenotipo.(10)

II. HIPÓTESIS DE TRABAJO Y OBJETIVOS

1. Hipótesis de trabajo

El linfoma difuso de células grandes (LDCGB) se engloba en una sola categoría en la clasificación de la OMS. Sin embargo, hay múltiples evidencias de que, en realidad, el LDCGB constituye una entidad heterogénea con diferencias inmunofenotípicas, genéticas y moleculares que traducirían un origen celular diferente. Desafortunadamente, las técnicas de diagnóstico actuales no son lo suficientemente precisas para categorizar de manera reproducible los diferentes tipos de LDCGB, particularmente los grupos moleculares centro-germinal y activado. Por otro lado, el mismo origen primario del linfoma (ganglionar o extraganglionar) puede también marcar diferencias sustanciales en los LDCGB. Estas características clínico-biológicas, difícilmente reproducibles, tienen importantes repercusiones en el pronóstico y tratamiento de estos enfermos, de manera que una subclasificación reproducible sería extraordinariamente importante en la práctica clínica. Para completar el panorama, otro aspecto a tener en cuenta es que los nuevos tratamientos del LDCGB basados en la

inmunoquimioterapia pueden modificar la importancia de estas variables en el devenir de los pacientes.

En este contexto, la hipótesis de trabajo planteada en el primer estudio fue que el LDCGB de origen primario extraganglionar podía tener características clínico-biológicas diferenciadas respecto a las formas de origen ganglionar, así como presentar un beneficio limitado del tratamiento con inmunoquimioterapia.

En el segundo trabajo, la hipótesis planteada fue que la reproducibilidad de los grupos moleculares del LDCGB basados originalmente en las técnicas de expresión génica, es baja cuando se utiliza la inmunohistoquímica, en especial en los pacientes tratados con inmunoquimioterapia. Ello sería muy relevante, dada la importancia pronostica y terapéutica que se ha dado a los subtipos moleculares durante la última década.

2. Objetivos

2.1. Primer trabajo

1. Analizar las principales características de los pacientes con LDCGB según el origen primario de la enfermedad (ganglionar frente a extraganglionar).

2. Analizar el impacto diferencial de la adición de rituximab a la poliquimioterapia convencional en los pacientes con linfoma primario ganglionar o extraganglionar.

2.2. Segundo trabajo

1. Comparar los diferentes algoritmos inmunohistoquímicos (Colomo, Hans, Muris, Choi y Tally), en una serie de pacientes con LDCGB tratados de manera homogénea con inmunoquimioterapia.

2. Correlacionar la información obtenida con los perfiles de expresión génica (GEP) y los diferentes algoritmos inmunohistoquímicos.

3. Analizar el valor pronóstico de los subtipos moleculares definidos por GEP, así como de los definidos mediante los diferentes algoritmos inmunohistoquímicos.

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III. RESULTADOS

1. Primer trabajo

"The impact of immunochemotherapy in the outcome of patients with primary nodal or extranodal diffuse large B-cell lymphoma"

Gonzalo Gutiérrez-García, Lluis Colomo, Neus Villamor, Leonor Arenillas, Antonio Martínez, Teresa Cardesa, Adriana García-Herrera, Xavier Setoain, Sonia Rodríguez, Gabriela Ghita, Pau Abrisqueta, Eva Giné, Francesc Bosch, Elías Campo, Emilio Montserrat, y Armando López-Guillermo

Leukemia & Lymphoma. 2010 Jul; 51(7): 1225-32.

Resumen

Aunque considerado como una categoría en la actual clasificación de la OMS, el LDCGB incluye diferentes entidades y variantes. En este sentido, una tercera parte de los LDCGB tiene un origen primario diferente al territorio ganglionar, habitualmente denominados linfomas extraganglionares. Si bien este grupo de linfomas no son reconocidos como una variante del LDCGB dentro de la clasificación de la OMS, son numerosas las publicaciones que describen las características clínicas y biológicas de este grupo. Por otro lado, la introducción de la inmunoquimioterapia con rituximab (IQT-R) en el tratamiento de los linfomas ha mejorado notablemente el pronóstico de los pacientes. Sin embargo, la eficacia de la IQT-R ha sido cuestionada en algunos casos. Los objetivos del estudio fueron evaluar las características clínico-biológicas del LDCGB según el origen primario (ganglionar frente a extraganglionar), y el impacto de la IQT-R en pacientes con LDCGB de acuerdo al origen del linfoma (extraganglionar frente a ganglionar). Para ello, se consideraron 262 pacientes diagnosticados con DLBCL en una institución, 5 años antes y después de establecerse la IQT-R como tratamiento estándar en el LDCGB. Ciento dieciséis pacientes (44%) recibieron tratamiento con el régimen CHOP y 146 (56%) fueron tratados con R-CHOP. Los pacientes que recibieron R-CHOP tuvieron una tasa de RC superior que aquellos tratados con CHOP (78% frente a 60%, respectivamente; p=0,002). En el análisis por grupos de acuerdo al origen primario (ganglionar frente a EG) y el tratamiento (CHOP frente a R-

CHOP) solo en el grupo ganglionar, la SG a 5 años fue superior de forma significativa (80% frente a 100%; p=0,001). En el análisis multivariado mientras que en el grupo de linfomas ganglionares el IPI (riesgo bajo frente a intermedio/alto) y el tratamiento (R-CHOP frente a CHOP) fueron variables predictoras de la SG, en el grupo de linfomas EG solo el IPI (riesgo bajo frente a intermedio/alto) fue una variable predictora de la SG.

En conclusión el origen primario del LDCGB (ganglionar frente a EG) se asoció de manera particular con características clínicas, biológicas y pronosticas. El uso de la inmunoquimioterapia con rituximab incrementó dramáticamente el pronóstico del LDCGB de origen ganglionar, mientras que su efecto fue mucho menor en los casos extraganglionares.

The impact of immunochemotherapy in the outcome of patients with primary nodal or extranodal diffuse large B-cell lymphoma

Abstract

In diffuse large B-cell lymphoma (DLBCL), immunochemotherapy effectiveness has been questioned in some cases. The aim of our study was to assess the impact of rituximab in patients with DLBCL according to the primary site (nodal vs. extranodal.). We reviewed 262 patients diagnosed with DLBCL in a single institution 5 years before and after immunochemotherapy was considered the standard. 116 patients received CHOP and 146 rituximab plus CHOP (R-CHOP). The primary site of the disease was lymph-node in 140 patients (53%), Waldeyer's ring (WR) in 22, gastro-intestinal (GI) in 33, and other extranodal in 67. The addition of rituximab significantly improved CR rate in nodal, but not in extranodal lymphomas. Patients receiving R-CHOP showed higher OS than those treated with CHOP alone (5-year OS: 71% vs. 48%). This difference maintained in primary nodal (5-year OS: 66% vs. 36%), but was not observed in WR (80% vs. 100%), GI (90% vs. 92%) or other primary extranodal DLBCL (67% vs. 58%). IPI, treatment and primary site were the main variables for OS in multivariate analysis. In nodal cases, IPI and treatment maintained the value, whereas only IPI predicted OS in extranodal cases. In conclusion, patients with extranodal DLBCL had limited benefit from the use of R-CHOP, observation that should be confirmed in prospective studies.

Introduction

Around one third of the non-Hodgkin's lymphomas arise in tissues other than lymph nodes, being usually termed extranodal lymphomas.(81-84) The incidence of lymphomas has increased during the last decades, with extranodal lymphomas increasing more rapidly than nodal.(83, 85) Although there are multiple publications in the literature dealing with etiopathogenesis, biological features, clinical characteristics and outcome of extranodal lymphomas, most of them include heterogeneous series of patients, in which several histologic subgroups are usually merged.(12, 13, 83, 84, 86-91) Diffuse large B-cell lymphoma [DLBCL] is the commonest type of non-Hodgkin's lymphoma in Western countries, representing around 30% of them.(1) About one third of DLBCLs have a primary extranodal origin. Although considered a single category in the REAL/WHO classification, DLBCL most likely includes different clinicopathologic entities that are currently difficult to separate with the standard techniques.(7, 74, 81, 92) In this setting, extranodal DLBCL might also have a specific personality.(15)

Since the incorporation of rituximab (an anti-CD20 chimeric monoclonal antibody) into the armamentarium against lymphomas, the combination of chemotherapy plus rituximab, so-called immunochemotherapy, has become the gold-standard treatment in DLBCLs.(2) Thus, up to 15-20% of improvement in overall survival [OS] has been demonstrated in different settings of patients with respect to chemotherapy alone.(50, 93-96) Nevertheless, the effectiveness of immunochemotherapy has been questioned in some cases, such as patients with no tumor

expression of bcl2, those with bcl-6 expression or those of germinal-center origin.(42, 57, 97-102) Very few information is available on the efficacy of immunochemotherapy in patients with extranodal DLBCL in large series of patients, although immunochemotherapy is considered the standard of care because of the global results.

The aim of the present study was to analyze the impact of the addition of rituximab to the standard CHOP regimen in the outcome of the patients from a single institution according to the primary site of the lymphoma (nodal vs. extranodal).

Patients and Methods

Patients

Three hundred sixty-eight patients consecutively diagnosed with a CD20-positive DLBCL between January 1997 and December 2006, and followed up in a single institution were selected for the present study. The cases with recognized disease phase of a follicular lymphoma or another type of indolent lymphoma with subsequent transformation into a DLBCL, primary mediastinal, intravascular, and primary effusion were not included. In addition, immunodeficiency associated tumors (patients HIV-positive, 41 cases; post-transplant lymphoproliferative disorders, 5 cases), and primary CNS lymphoma (12 cases) were excluded from the study. Finally, 48 patients who received non-adriamycin-containing chemotherapy for different reasons were also excluded. Thus, the remainder 262 patients constituted the subjects of the present study.

Median age of the patients was 60 years (range, 19 to 81) and the male/female distribution was 134 / 128. Main initial characteristics of the patients are listed in Table 5. Advanced stage (Ann Arbor III or IV) was observed in 140 cases (55%), and any extranodal involvement in 181 (69%), including bone marrow infiltration in 52 cases (20%). 133 patients of 244 with available data (55%) had high serum lactic acid dehydrogenase [LDH] levels, whereas the distribution according to the International Prognostic Index [IPI] was the following: low-risk, 90 cases (37%); low/intermediate, 41 cases (17%); high/intermediate, 51 cases (21%); high-risk, 62 cases (25%); and non assessable, 18 cases. The main initial and evolutive variables,

including the histologic parameters below indicated, were recorded and analyzed for prognosis.

Staging maneuvers included patient history and physical examination (including Waldeyer's ring [WR] area), blood cell counts and serum biochemistry, including LDH and β2-microglobulin [β2m] levels, computerized tomography scan of chest, abdomen and pelvis, as well as bone marrow biopsy.

Treatment

The period of time of the study was five years before (1997-2001) and after (2002-2006) rituximab-containing chemotherapies were established as the standard treatment for patients with DLBCL. Thus, before January 2002, 116 patients (44%) received the regimen CHOP (cyclophosphamide, adriamycin, vincristine and prednisone) and, after that time, 146 (56%) patients were treated with CHOP plus rituximab (R-CHOP). The distinction between nodal and extranodal was not taken into account to plan the treatment. No significant differences were found in the main initial features of the patients according to the treatment given (CHOP vs. R-CHOP) (data not shown). Post-therapy re-staging consisted of the repetition of the previously abnormal tests and/or biopsies. Response was assessed according to conventional criteria.(103) Overall, 177 patients achieved a complete response [CR] (69%), 28 patients a partial response (11%), and 50 (20%) patients failed to treatment. In 7 cases the response was not assessable. After a median follow-up of 4.9 years (range, 0.2 to 11.9) for surviving patients, 112 patients have died. The 5-year and 10-year overall survival was 60% (95% confidence interval [CI]: 54–66%) and 53% (95%CI:

45-61%), respectively (Figure 18A). Median follow up for patients receiving CHOP and R-CHOP were 8.5 and 3.7 years, respectively.

	Nodal (N=140)	*Waldeyer's Ring (N=22)*	*Gastro-intestinal (N=33)*	*Other extranodal (N=67)*
Gender (M/F)	67 / 73	12 / 10	19 / 14	36 / 31
Age (median; range)	60 (19-81)	65 (26-80)	54 (24-78)	60 (21-81)
B-symptoms (%)	41	9*	28**	34
ECOG ≥2 (%)	46	9*	31	45
Advanced stage (%)	61	23*	30**	64
Bone marrow (+) (%)	26	9*	0**	22
High serum LDH (%)	59	43	33**	59
High serum β2m (%)	55	15*	33	49
IPI (risk)				
low (%)	30	71	58	30
low/intermediate (%)	20	0	14	17
high/intermediate (%)	26	24	7	15
high (%)	24	5*	21**	38

Table 5. Main clinical features at diagnosis of 262 patients with DLBCL according to the primary site of the disease. *p<0.01 vs. the other groups; **p<0.05 vs. the other groups. The number of patients with available data for ECOG, bone marrow, LDH, β2m, and IPI were 255, 260, 244, 216 and 244, respectively.

Nodal / extranodal definitions

Lymphomas arising in extranodal organs, with no or only minor lymph node involvement, were considered as primary extranodal. Lymphomas with lymph node involvement clinically dominant, as well as those presenting at spleen were considered as primary nodal. Lymphomas from the Waldeyer's ring, although considered nodal, were analyzed separately. Finally, those lymphomas with extensive disease involving both nodal and extranodal sites were considered as nodal.

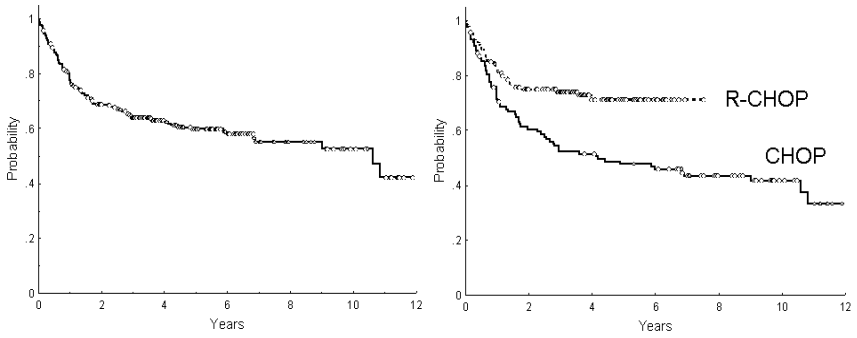

Figure 18. (A) Overall survival (OS) of 262 patients with diffuse large B-cell lymphoma treated with curative intention; (B) OS of the same 262 patients according to the therapy given: CHOP vs. rituximab plus CHOP (R-CHOP).

Histologic features

The diagnosis of DLCL was based in all the cases on the criteria established in the WHO classification.(81) For the morphologic analysis, all the histologic slides were reviewed by three different observers (AM, LC & EC). The panel of monoclonal antibodies included antibodies against the following antigens: CD20, CD79a, CD3, CD5, CD10, MUM1/IRF4, CD138, bcl-2, bcl-6, p53 and p27. The proliferative index was assessed by Ki-67 immunostaining. The antibodies and the immunohistochemical conditions of use have been previously described.(36) The patients were assigned to germinal center B-cell like (GCB) or non-GCB groups according to methods previously described. (36, 79)

Statistical análisis

Categorical data were compared using Fisher's exact test, two-sided *P* value, whereas for ordinal data non-parametric tests were used. The multivariate analysis of the variables predicting

response was performed by using a logistic regression. The definitions of CR, disease-free survival [DFS] and overall survival [OS] were the standard (104). The actuarial survival analysis was performed according to the method described by Kaplan and Meier(105) and the curves compared by the log-rank test.(106) The multivariate analysis for survival was performed by using the stepwise proportional hazards model (Cox).(107)

Results

Distribution and clinicobiological features

One hundred patients (38%) presented at a primary extranodal site, 22 patients (9%) at WR, whereas the remainder one hundred forty (53%) had a primary nodal DLBCL, including one lymphoma of the spleen. The distribution according to the extranodal site was the following: gastrointestinal [GI] tract (33 cases, 12% of the overall series; with gastric lymphoma found in 30 cases), soft tissue (16 cases; 6%), breast (13 cases; 5%), lung and pleura (11 cases; 4%), liver (9 cases; 3%), bone (9 cases; 3%), kidney (3 cases), testis/ovary (2 cases), bone marrow (2 cases), skin and thyroid (1 case each).

The main clinical features at diagnosis according to the primary site of the lymphoma are detailed in Table 5. Patients with WR DLBCL more frequently presented with early stage, ambulatory performance status, absence of B-symptoms, normal serum albumin and β2-m levels, and low- or low/intermediate-risk IPI ($p<0.01$ in all cases *vs.* the other groups). GI DLBCL also had more frequently an early stage, absence of bone marrow infiltration, normal serum LDH and low- or low/intermediate-risk IPI than the other groups ($p<0.05$ in all the cases). The immunohistochemical expression of the main single antigens according to the origin of the lymphoma is summarized in Table 6. Patients with primary extranodal lymphoma more frequently presented with bcl-6 expression than those with a lymphoma of nodal origin (13/22 [59%] *vs.* 37/60 [67%], respectively; $p=0.04$).

No significant differences were found regarding to the remainder antigens or the differentiation profile.

Response to treatment

No differences were found in the treatment given to the patients (CHOP vs. R-CHOP) according to the primary origin of the lymphoma. One hundred seventy seven of 255 assessable patients (69%) achieved CR. Patients with lymphoma of WR (19/21, 90%) and GI (29/32, 91%) showed a higher CR rate than the others. Patients receiving R-CHOP showed a higher CR rate than those treated with CHOP (78 vs. 60%, respectively; p=0.002). Moreover, the proportion of primary refractory patients was significantly lower in those patients treated with R-CHOP than in those receiving CHOP (16 vs. 23%, respectively; p=0.002). In Table 7, the differences in CR rate and the proportion of primary refractory patients are detailed for the different nodal and extranodal groups. Whereas in nodal lymphomas the addition of rituximab significantly improved CR rate and decreased the proportion of refractory patients, no significant difference was found between therapy with CHOP or R-CHOP in primary extranodal lymphomas. The following variables predicted for CR achievement: age <60 years, ambulatory performance status (ECOG<2), absence of B-symptoms, absence of bulky disease, early Ann Arbor stage, no bone marrow involvement, normal serum albumin, LDH and β2m levels, as well as the IPI and treatment with R-CHOP. In the logistic regression analysis, IPI (p<0.001; relative risk [RR]: 0.13) and R-CHOP (p=0.003; RR: 2.56) were the most important variables to predict CR achievement. In nodal DLBCL patients, IPI (p<0.001; RR: 0.32)

and R-CHOP (p=0.03; RR: 2.5) maintained the predictive value. In primary extranodal cases only IPI (p<0.003; RR: 0.18) and bulky disease (p=0.04; RR: 0.31) had importance for CR, whereas R-CHOP lost its prognostic value.

	Nodal (N=64)	*Waldeyer's ring (N=13)*	*Gastro-intestinal (N=12)*	*Other extranodal (N=30)*
Bcl-2 expression (%)	60	80	20	50
CD10+ (%)	37	10	67	44
Bcl-6+ (%)	58	80	80	53
MUM-1+ (%)	58	78	25	40
Differentiation profile*				
Germinal-center (%)	42	30	66	50
Non-germinal-center (%)	58	70	34	50

Table 6. Immunophenotypic features of the 78 patients with diffuse large B-cell lymphoma in whom adequate material was available to assess CD10, bcl-6, MUM-1 and bcl-2 expression, according to the primary site of the disease.

No single antigen expression or differentiation profile predicted CR achievement. Both germinal-center and non-germinal-center lymphomas benefit from the addition of rituximab, irrespective of the nodal or extranodal primary site.

Disease-free survival

Forty-two of 177 CR patients eventually relapsed, with a 5-year DFS of 75% (95% CI: 68-82%). The DFS according to the primary site of the lymphomas is detailed in Table 7. Patients with primary extranodal lymphoma showed a higher DFS than those with nodal disease (5-year DFS: 83 vs. 69%, respectively; p=0.02). Overall, patients receiving R-CHOP did not have a significantly longer DFS than those treated with CHOP alone (5-year DFS: 76 vs. 70%, respectively; p=NS). However, when

analyzing by subgroups, as indicated in Table 7, primary nodal lymphomas, excluding WR, treated with R-CHOP showed a significantly higher DFS than those treated with CHOP alone (5-year DFS: 74 vs. 56%, respectively; p=0.05). DFS curves are depicted in Figure 19.

	CR rate		*Primary refractory*		*Early death*		*5-year DFS*		*5-year OS*	
	CT (%)	R-CT (%)	CT (%)	R-CT (%)	CT (%)	R-CT (%)	CT (%)	R-CT (%)	CT (%)	R-CT (%)
Nodal (N=140)	51 *	75	33 *	18	9	8	60 *	75	36 *	66
Waldeyer's ring (N=22)	100	86	0	0	0	0	100	85	100	80
Gastrointestinal (N=33)	92	90	0	0	0	0	75	76	92	90
Other extranodal (N=67)	59	72	17	16	14	8	76	84	58	67

Table 7. Response to treatment, early death, disease-free survival (DFS) and overall survival (OS) of 262 patients with diffuse large B-cell lymphoma according to the treatment given (chemotherapy [CT] vs. rituximab plus chemotherapy [R-CT]) for the different primary sites of the disease. *p<0,05

Overall survival

One hundred and five patients have died during the follow-up. Twenty seven patients died within 4 months from diagnosis and were considered as "early deaths". The treatment with R-CHOP did not influence early deaths neither in the whole series nor in the primary nodal or extranodal subsets. Five-year OS of the entire series was 60 % (95% CI: 54 – 66%). Unfavorable variables predicting OS were: age >60 years, presence of B-symptoms, poor performance status (ECOG≥2), advanced Ann Arbor stage (III-IV), extranodal involvement ≥2 sites, bone marrow involvement, anemia (hemoglobin <12 g/L),

thrombocytopenia (platelet count <100x10^9/L), high erythrocyte sedimentation rate (>40 mm/h), low serum albumin levels, high serum LDH levels, and high β2-m. In addition, bcl-2 protein expression and absence of bcl-6 expression predicted poor OS. IPI also had a high value to predict OS. In the whole series, there was a significant advantage in terms of OS of patients treated with R-CHOP (5-year OS, 48 vs. 71% for CHOP and R-CHOP, respectively; p<0.001) (Figure 18B).

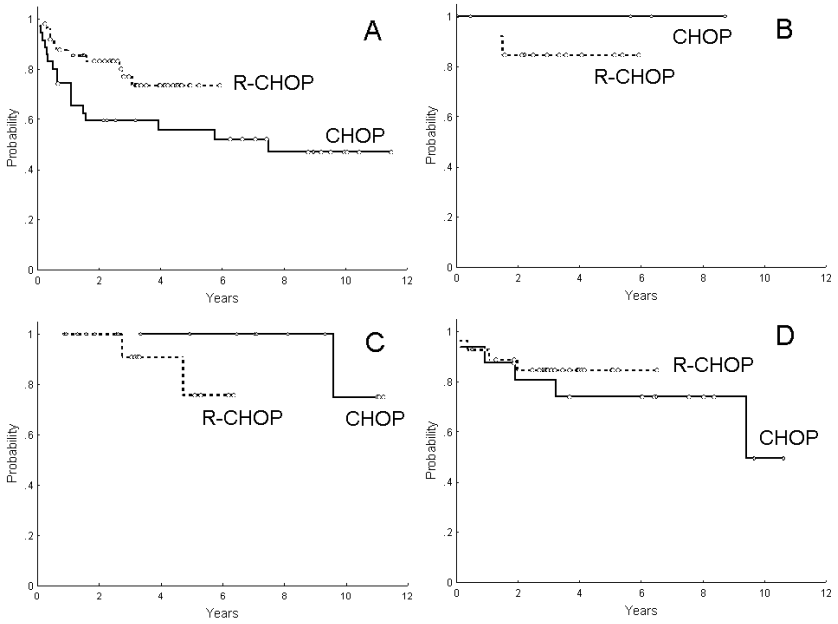

Figure 19. Disease-free survival of patients with diffuse large B-cell lymphoma according to the therapy given, CHOP vs. rituximab plus CHOP (R-CHOP) and to the primary site of the disease: A) lymph node (N=140), P=0.05; B) Waldeyer's ring (N=22), P=0.5; C) gastrointestinal (N=33), P=0.1; D) other extranodal (N=67), P=0.5

The effect of rituximab-containing treatment among different subsets of nodal and extranodal lymphomas is detailed in Table 7 and depicted in Figure 20. Thus, patients treated with R-CHOP showed longer OS than those receiving CHOP alone only in

primary nodal cases, excluding Waldeyer's (5-year OS: 66 vs. 36%, respectively; p=0.001).

To further assess the impact of rituximab-containing regimens in OS according to the different primary sites of presentation a multivariate analysis was performed including the most significant variables predicting OS in the univariant analysis, along with the primary site of the lymphoma (nodal vs. extranodal) and the therapy given (CHOP vs. R-CHOP).

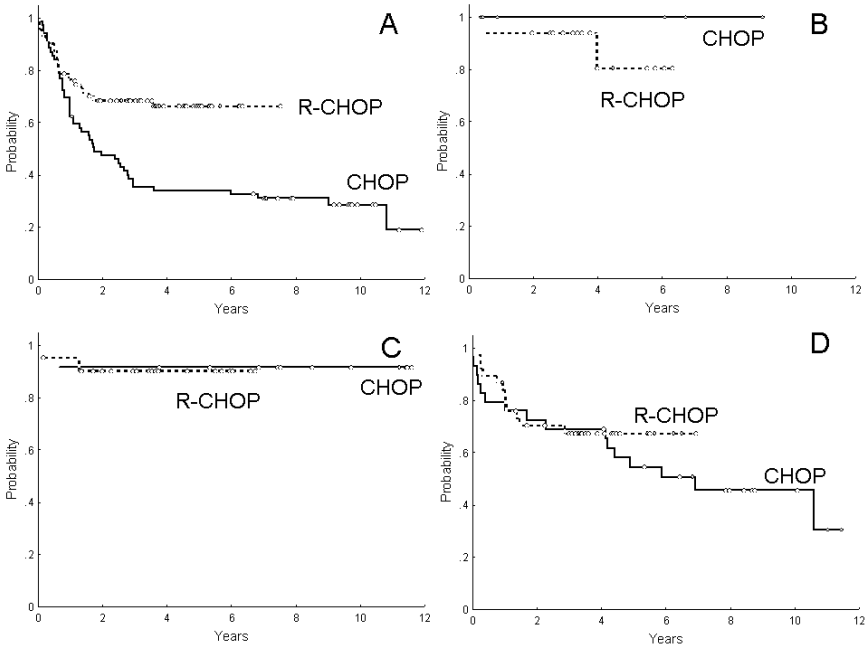

Figure 20. Overall survival of patients with diffuse large B-cell lymphoma according to the therapy given, (CHOP vs. R-CHOP) and to the primary site of the disease: A) lymph node (N=140), P=0.01; B) Waldeyer's ring (N=22), P=0.4; C) gastrointestinal (N=33), P=0.9; D) other extranodal (N=67), P=0.5

In the whole group, in the final model with 243 assessable patients, IPI (low vs. intermediate vs. high-risk; RR: 4.0 and 1.96; p<0.001), treatment (CHOP vs. R-CHOP; RR: 0.55; p=0.005) and primary site of the lymphoma (nodal vs. extranodal; RR: 0.57; p=0.01) were the most important variables

to predict OS. This analysis was performed in primary nodal and extranodal subgroups. In patients with nodal lymphomas, IPI (RR: 3.25 and 1.35; p=0.01) and treatment (RR: 0.41; p=0.001) maintained the prognostic value for OS, whereas in primary extranodal cases only IPI (RR: 5.58 and 4.9; p<0.001) was important for OS.

In the subset of 78 patients with this information available neither the expression of single antigens, or the GC, or non GC cell of origin profile predicted OS before or after R-CT. The primary sites did not significantly influenced OS in the different subsets.

Discussion

The primary site of the lymphoma, either the lymph node or different extranodal territories, can separate two different groups of DLBCLs, nodal and extranodal, with particular clinicobiological features and different natural history.(15) It has been suggested that genetic differences between nodal and extranodal DLBCLs might exist, including single gene alterations, such as *c-MYC*, *BCL-6*, *REL* and *FAS* (more frequently seen in extranodal DLBCL).(14) However, no specific alteration has been described and, for the moment, the WHO classification does not take into consideration the primary site of the lymphoma for the classification.

The consideration of a lymphoma as primary nodal or extranodal is controversial.(12) Patients with purely nodal or extranodal involvement are easily classified. Although in some studies, only localized extranodal lymphomas have been defined as primary extranodal,(87, 108) this restrictive criterion gives to an incomplete picture of these lymphomas. For this reason, those cases with "clinically relevant" extranodal involvement are usually considered as extranodal.(12, 13, 15, 82-84) The cases with extensive disease, involving both nodal and extranodal areas, are difficult to categorize. In the present report, following previous publications, these cases were included among the nodal lymphomas.(12) This may represent a bias against the nodal group, but does not affect to the activity of therapy in the extranodal subgroup.

For decades, the treatment of patients with DLBCL was based on chemotherapy. The addition of rituximab to chemotherapy, the

so-called immunochemotherapy, dramatically improved the outcome of these patients, as demonstrated both in clinical trials and in retrospective population-based studies.(50, 93-96) Thus, at present time, immunochemotherapy is considered the gold-standard treatment for any CD20-positive DLBCL.(2) However, since DLBCL is heterogeneous at molecular level, some authors hypothesize that rituximab improve survival mostly in certain subgroups of DLBCLs. In fact, different reports have pointed out that the positive effect of adding rituximab might be only marginal in specific subsets of patients. For instance, there are data supporting that only patients with bcl-2-positive tumor (but not those bcl-2-negative)(57, 98, 99) or only patients bcl-6-negative (bur not those bcl-6-positive)(42) significantly benefit from immunochemotherapy. Moreover, patients with ABC-DLBCL seemed to benefit much more of adding rituximab than patients with CG-DLBCL.(101) Certainly, these observations should be confirmed in prospective larger series and, in fact, recently published data seem to disprove some of these assertions.(102, 109)

The present study confirms that the use of immunochemotherapy dramatically improved both CR rate and OS in an unselected series of DLBCL patients from a single institution. Interestingly, when analyzing the results according to the different primary sites of the disease, we found intriguing results. Thus, patients with primary extranodal lymphomas showed no clear benefit to the addition of rituximab in terms of response, DFS and OS. This was true for the different sites analyzed (WR, GI and other sites). Of note, these finding were not related to the initial risk of the patient, as measured by Ann Arbor stage or IPI. In addition,

although the information is limited due to the relative small number of patients studied, no biological parameter was related to this lack of efficiency of immunochemotherapy. The information in the literature on the activity of immunochemotherapy in extranodal lymphomas is scarce.(110) No large clinical trial has been performed in this setting of patients.

Several reasons could be invoked to explain the present findings. The subset analysis tends to fractionate the series in small groups, in which it would be more difficult to reach statistical significance. However, this is not the case, since as depicted in Figures 19 and 20 it is not a matter of numbers, but of lack of real differences between the groups. On the other hand, patients with WR and GI DLBCLs had so good outcome when treated with CT alone that was very difficult to demonstrate an improvement of the results. Certainly, the number of patients with extranodal DLBCL in this series is not powered to discard a benefit of rituximab. Nevertheless, it must be indicated that in most series of DLBCL the greatest improvement of outcome has been mainly observed in low-risk patients.(93) More interestingly, the relative lack of effectiveness of R-CHOP in extranodal DLBCLs could reflect real biological particularities of these tumors. Although we have not been able to show differences in terms of single antigen expression, including bcl-2 and bcl-6, or differentiation profile, only immunohistochemical studies could be performed, since the available material was not adequate for microarrays technology. In this regard, a recent publication has stressed the importance of the microenvironment in tumor invasion, disease progression and clinical outcome of DLBCL patients. Two gene-expression

signatures, stromal-1 or stromal-2, that reflect the character of non-malignant cells, have been able to distinguish prognostic subsets in DLBCL with independent value from GCB or ABC origin. No information is available on how the microenvironment could affect the response to immunochemotherapy (all the patients had been treated with R-CHOP)(109, 111). In addition, there are no data of how the stromal gene signatures could be differentially expressed in nodal and extranodal DLBCLs.

In conclusion, in the present series the patients with primary extranodal DLBCL seem to have little benefit from the use of immunochemotherapy. Of course, these data could not change the treatment guidelines in patients with extranodal DLBCL, but this intriguing observation should be confirmed in further prospective studies.

2. Segundo trabajo

"Gene expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy"
"

Gonzalo Gutiérrez-García, Teresa Cardesa-Salzmann, Fina Climent, Eva González-Barca, Santiago Mercadal, José L. Mate, Juan M. Sancho, Leonor Arenillas, Sergi Serrano, Lourdes Escoda, Salomé Martínez, Alexandra Valera, Antonio Martínez, Pedro Jares, Magdalena Pinyol, Adriana García-Herrera, Alejandra Martínez-Trillos, Eva Giné, Neus Villamor, Elías Campo, Luis Colomo, y Armando López-Guillermo, *Grup per l'Estudi dels Limfomes de Catalunya I Balears* (GELCAB)

Blood. 2011 May 5;117(18):4836-4843

Resumen

Los estudios de expresión génica (GEP) han permitido separar al linfoma difuso de células grandes (LDCGB) en dos subtipos, aquellos de origen centrogerminal (LDCGB-CGB) y los de origen activado (LDCGB-ABC), teniendo este último grupo un pronóstico desfavorable.(112) Este hallazgo pronóstico, en relación al origen del LDCGB inicialmente descrito en la era pre-rituximab, se ha confirmado recientemente en series de pacientes tratados con inmunoquimioterapia con rituximab (IQT-R). Sin embargo, las técnicas de GEP no son aplicables de manera rutinaria a la práctica clínica. La posibilidad de remedar los resultados de GEP mediante el uso de algoritmos inmunohistoquímicos es de gran interés clínico. Tres de estos algoritmos fueron elaborados en la era pre-rituximab, y otros dos en la era de la inmunoquimioterapia. Sin embargo, la fiabilidad de la inmunohistoquímica como remedo de los GEP es controvertida. En este contexto, los objetivos del estudio fueron comparar entre sí los diferentes algoritmos inmunohistoquímicos (Colomo, Hans, Muris, Choi y Tally), en una serie de pacientes con LDCGB tratados de manera homogénea con inmunoquimioterapia. Además, establecer la correlación entre la información obtenida con los GEP y los diferentes algoritmos inmunohistoquímicos. Se analizó el valor pronóstico de los GEP y de los diferentes algoritmos inmunohistoquímicos. Para ello, se construyeron perfiles de expresión de tejido con muestras de 157 pacientes con LDCGB tratados de forma homogénea con inmunoquimioterapia, con el fin de aplicar los algoritmos

siguientes: Colomo (MUM1/IRF4, CD10 y antígenos BCL6), Hans (CD10, BCL6 y MUM1/IRF4), Muris (CD10, MUM1/IRF4 y BCL2), Choi (GCET1, MUM1/IRF4, CD10, FOXP1 y BCL6) y Tally (CD10, GCET1, MUM1/IRF4, FOXP1 y LMO2). En 62 casos tuvimos información de expresión génica (GEP). La proporción de casos mal clasificados por la inmunohistoquímica, en comparación con el GEP fue mayor cuando se definió el subgrupo CGB: 41%, 48%, 30%, 60% y 40% para Colomo, Hans, Muris, Choi y Tally, respectivamente. La supervivencia libre de progresión y la supervivencia global (SG) de la serie global fueron 50% y 58%, respectivamente. En el estudio multivariante, el origen ABC del linfoma fue la variable que mejor predijo la SG. Por el contrario, ninguno de los algoritmos fue predictor de la SG. Con estos resultados podemos concluir en el presente estudio que ninguno de los 5 algoritmos inmunohistoquímicos fue capaz de remedar los GEP o de separar el valor pronóstico de los grupos moleculares. Por lo tanto, hoy en día una clasificación del LDCGB basada en la inmunohistoquímica (CGB frente a no-CGB) y cuyo fin sea terapéutico, debe ser considerada muy cautelosamente, aún en ensayos clínicos.

Gene expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy

Abstract

Diffuse large B-cell lymphomas (DLBCL) can be divided into germinal-center B-cell-like (GCB) and activated-B-cell-like subtypes by gene expression profiling (GEP), with the latter showing a poorer outcome. Although this classification can be mimicked by different immunostaining algorithms, their reliability is object of controversy. In this setting, we constructed tissue microarrays with samples of 157 DLBCL patients homogeneously treated with immunochemotherapy in order to apply the following algorithms: Colomo (MUM1/IRF4, CD10 and BCL6 antigens), Hans (CD10, BCL6 and MUM1/IRF4), Muris (CD10 and MUM1/IRF4 plus BCL2), Choi (GCET1, MUM1/IRF4, CD10, FOXP1 and BCL6) and Tally (CD10, GCET1, MUM1/IRF4, FOXP1 and LMO2). GEP information was available in 62 cases. The proportion of misclassified cases by immunohistochemistry as compared with GEP was higher when defining the GCB subset: 41%, 48%, 30%, 60% and 40% for Colomo, Hans, Muris, Choi and Tally, respectively. Whereas the GEP groups showed significantly different 5-year progression-free survival (76% vs. 31% for GCB and activated DLBCL) and overall survival (80% vs. 45%), none of the immunostaining algorithms was able to retain the prognostic

impact of the groups (GCB vs. non-GCB). In conclusion, stratification based on immunostaining algorithms for guiding therapy should be cautiously considered, even in clinical trials.

Introduction

Diffuse large B-cell lymphoma (DLBCL), although considered a single category in the WHO classification, most likely includes different clinicopathologic entities difficult to separate with the standard techniques.(10, 74) From the clinical standpoint, the introduction of the immunochemoterapy in the treatment of DLBCL has dramatically improved the outcome of these patients with respect to chemotherapy alone.(50, 54, 55, 93, 94, 113) However, a significant proportion of these patients (20-30%) is refractory or eventually relapses.(16) Nowadays, the identification of factors, either biological or clinical, that could recognize poor risk patients is a priority. Different prognostic factors for response and survival have been described in DLBCL, but in the rituximab era, the role of the biological prognostic factors is yet to be determined.(20)

DLBCLs can be divided by means of gene expression profile (GEP) studies into germinal center B-cell-like (GCB) and activated B-cell-like (ABC) subtypes, with the latter having a significantly poorer outcome than the GCB group.(75) These molecular subtypes are associated with a different outcome of patients, even after the introduction of immunochemotherapy.(49) However, GEP techniques are not applicable to the routine clinical practice and different approaches using immunophenotypic algorithms with small panels of biomarkers have been assayed, in order to translate the robust information of the molecular studies into a routine clinical platform.(36, 47, 48, 79, 80) Two of these algorithms were

designed in the pre-immunotherapy era and the other three were used in cohorts of patients treated with rituximab. The biomarkers used included the germinal center (GCB) markers (CD10, BCL6, GCET1 and LMO2) and antigens related to post-germinal center (non-GCB) differentiation such as MUM1/IRF4 and FOXP1. In addition, the categorization of DLBCL to a GCB or non-GCB subgroup was associated with clinicopathological features of the tumors and able to predict the outcome of the patients in some studies but not in others.(36, 37, 45, 47, 48, 70, 79, 80, 101, 102, 114) The causes of these contradictory results may be complex and include difficulties in the standardization of both the staining methodologies and evaluation of the results(115) and certain heterogeneity in the characteristics of the patients in different studies.(36, 37, 47, 48, 79, 80) However, it is not clear whether any of the algorithms is superior to others in obtaining the GEP molecular information since a comparative study using all of them has not been performed and only three studies have correlated the immunophenotypic algorithm with the GEP molecular classification.

The aim of the present study was to compare in this setting the above mentioned algorithms in a series of patients with DLBCL homogeneously treated with immunochemotherapy in order to assess the correlation of GEP data as well as their usefulness to predict patients' outcome.

Patients and Methods

Patients

Two-hundred eighty seven patients were diagnosed with DLBCL from January 2002 to December 2006 in 5 institutions from the *Grup per l'Estudi dels Limfomes de Catalunya i Balears* (GELCAB). The cases with recognized disease phase of a follicular lymphoma or another type of indolent lymphoma with subsequent transformation into a DLBCL, as well as immunodeficiency-associated tumors, post-transplant lymphoproliferative disorders, intravascular, central nervous system, primary effusion lymphomas and primary mediastinum lymphoma were excluded from the study. In 157 of the 287 patients the material necessary to construct a tissue microarray (TMA) and to assess the different antigen expression was available. These patients constituted the subjects of the present study. No significant differences were observed regarding main initial features and outcome between the 157 patients with available TMA and the remainder (data not shown).

Staging measures included patient history and physical examination, blood cell counts and serum biochemistry, including lactate dehydrogenase [LDH] and β2-microglobulin [β2m] levels, chest, abdomen and pelvis computerized tomography scan, as well as bone marrow biopsy. Post-therapy re-staging consisted of the repetition of the previously altered test and/or biopsies. Response was assessed according to conventional criteria.(116)

Median age of the patients was 65 years (range, 17 to 91) and the male/female distribution was 77 / 80. Main characteristics of the patients are listed in Table 8. All patients received rituximab-

containing chemotherapies, including regimens with anthracyclines in 133 (85%) patients. Response to treatment was as follows: complete response (CR), 119 (76%), partial response, 4 (2%), and failure to therapy 34 (22%) cases. Median follow-up of surviving patients was 4.3 years (range, 0.8 to 8.6). Five-year progression free-survival (PFS) of the series was 50% (95% CI: 42-58%). Sixty one patients died during the follow up with a 5-year overall survival (OS) of 58% (95%CI: 50-66%). PFS and OS curves are shown in Figure 21. The main initial and evolutive variables, including the histological parameters indicated below, were recorded and analyzed for prognosis.

Histological review and tissue microarray (TMA) construction

The diagnosis of DLBCL was based on the criteria established in the WHO classification.(10) The diagnostic samples were reviewed by expert hematopathologists of the five hospitals of the GELCAB involved in the study. All the immunohistochemical study was performed after constructing tissue microarrays (TMA) including two 1 mm representative cores of each case using a tissue arrayer (MTA I, Beecher Instruments Inc, WI, USA). Standardized methods for tissue fixation (10% buffered formalin) and processing were used at all participating centers. The immunostains were performed on formalin-fixed, paraffin-embedded tissue sections using a fully automated immunostainer (Bond Max, Vision Biosystems, Mount Waverley, Australia). All the immunostains were centralized in one of the centers involved in the study and evaluated in common in a multiheaded microscope.

Initial and evolutive features	
Age, median (range) ≤ 60 years	65 (17-91) 65 (41%)
Gender Female Male	 80 (51%) 77 (49%)
Poor performance status (ECOG>1)	66 (42%)
B – symptoms	55 (35%)
Extranodal involvement	63 (40%)
Bone marrow involvement	46 (29%)
Ann Arbor stage III-IV	94 (60%)
High serum LDH	93 (59%)
High serum β2-microglobulin*	64 (47%)
International prognostic index (risk) Low Low/intermediate High/intermediate High	 47 (30%) 35 (22%) 38 (25%) 37 (23%)
Response to therapy Complete response Partial response Non response/progression	 119 (76%) 4 (2%) 34 (22%)

Table 8. Initial and evolutive features of 157patients with diffuse large B-cell lymphoma included in the present study.

The conditions and antibodies are shown in Table 9. CD10, BCL6, MUM1/IRF4, BCL2, FOXP1, GCET1 and LMO2 were the markers used to build the algorithms. The assessment of CD10, BCL6, MUM1/IRF4 and BCL2 was performed according to the original papers describing the algorithms and following the recent guidelines recommended for their interpretation by the Luneburg Lymphoma Biomarker Consortium. Thus, appropriate internal controls were necessary to evaluate the immunostains.(117) FOXP1, GCET1 and LMO2 were evaluated

based on previous publications.(118-120) Endothelial cells were used as internal controls for FOXP1 and LMO2. For GCET1, tonsil sections were used as external control. The diagnostic samples and the immunohistochemical study were reviewed by at least three expert hematopathologists, blinded for the clinical details. The cores were evaluated in a semiquantitative manner and all markers used to build the algorithms were dichotomized at a positive cut-off that depended on the algorithm used. Small biopsy samples, which could not be included in a TMA, as well as cores which dropped off the TMAs were studied as whole tissue sections. For individual cores with discordant results, the core with representative and highest number of positive cells was scored. The discrepancies between the observers were resolved by reaching consensus. In addition to the markers included in the immunophenotypic algorithms we also studied the new GC markers HGAL, IRF8 and JAW1 and the non-GC markers XBP1 and BLIMP1. These additional biomarkers were evaluated semi-quantitatively and the samples were stratified into 5 groups: 1 (0% to fewer than 10% of positive tumor cells), 2 (10% to 25% positive cells), 3 (26% to 50% positive cells), 4 (51% to 75% positive cells), and 5 (more than 75% positive cells). The positive values and cut-off for these new markers was assessed based on previous studies.(120-123)

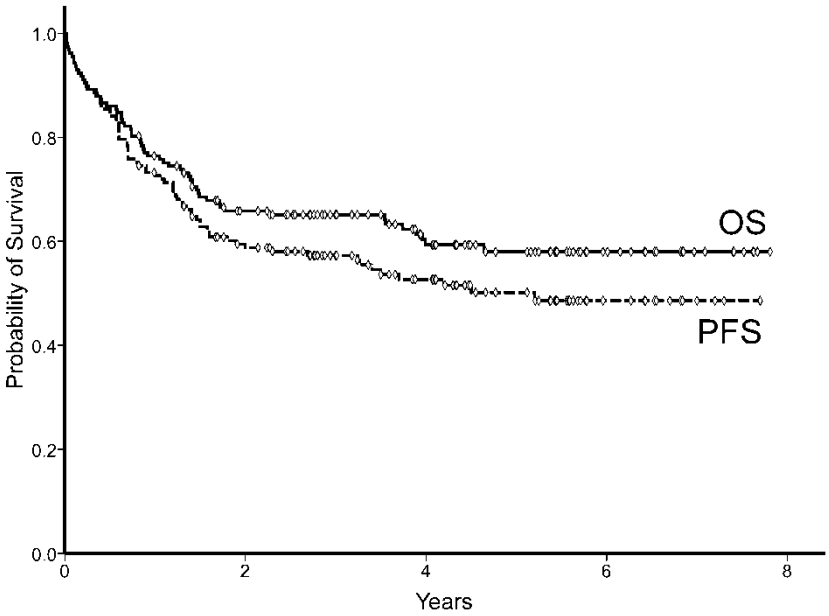

Figure 21. Progression-free survival (PFS) and overall survival (OS) of 157 patients with diffuse large B-cell lymphoma.

Immunophenotypic algorithms

To determine the origin of GCB or non-GCB, we applied five different algorithms previously published using the following panel of antibodies: CD10, BCL6, MUM1/IRF4, GCET1, FOXP1, LMO2 and BCL2. The thresholds used were the previously described in the literature for each algorithm and are detailed in Figure 22. In Colomo's algorithm (Figure 22A) the non-GCB phenotype was established if MUM1/IRF4 was positive. The cases MUM1/IRF4 negative and positive for CD10 were considered as GCB phenotype. Tumors MUM1/IRF4 and CD10 negative and BCL6 positive were also assigned to the GCB group. Finally, cases negative for the three markers were considered as not classified.(36) For Hans' algorithm (Figure 22B), the cases were assigned to the GCB phenotype if CD10 alone or both CD10 and BCL6 were positive.

Antigen	Source	Dilution	Incubation
CD20/CD79a	Dako	1/80	30 min
CD10	Novocastra	1/25	30 min
BCL6	CNIO*	1/25	60 min
GCET1	CNIO*	1/1	30 min
HGAL	Dako	1/500	60 min
IRF8	Sta Cruz Biotech Sc 13043 rabbit polyclonal	1/100	60 min
LMO2	Sta Cruz Biotech Sc 65736 mouse monoclonal	1/4000	60 min
JAW1	Sta Cruz Biotech Sc 11688 goat polyclonal	1/100	60 min
MUM1/IRF4	Dako	1/400	90 min
FOXP1**	A.H. Banham	1/80	30 min
BLIMP1	Sta Cruz Biotech Sc 13206	1/2	60 min
XBP1	Sta Cruz Biotech Sc 7160	1/400	150 min
BCL2	Dako	1/75	60 min

Table 9. Immunophenotyping study: antibodies and conditions of use

If both CD10 and BCL6 were negative, the case was considered as non-GCB origin.(79) In the Muris' algorithm (Figure 22C), CD10 positive cases were assigned to the GCB phenotype. CD10 negative cases were differentiated according to MUM1/IRF4 expression into ABC or GCB phenotype. Thereafter, BCL2 immunostaining was used to separate two different prognostic groups (1 and 2).(80) The two steps of Muris' algorithm to asses the GCB vs. ABC status and the prognostic impact of the algorithms was applied. In Choi's algorithm (Figure 22D) the cases positive for MUM1/IRF4 and/or FOXP1 or negative for CD10 and BCL6 were assigned to the non-GCB group. The cases positive for CD10, GCET1 without MUM1 expression, or BCL6 positive without FOXP1 expression were classified as GCB

group.(48) In Tally algorithm (Figure 22E) recently described, the method included an equal number of GCB markers (GCET1 and CD10) and non-GCB markers (FOXP1 and MUM1/IRF4). This algorithm is constructed from the immunophenotype pair with more positive antigens. Because two antibodies are used for each type, the LMO2 antigen determines the phenotype (GCB or non-GCB) when the score is equal in the two categories.(47)

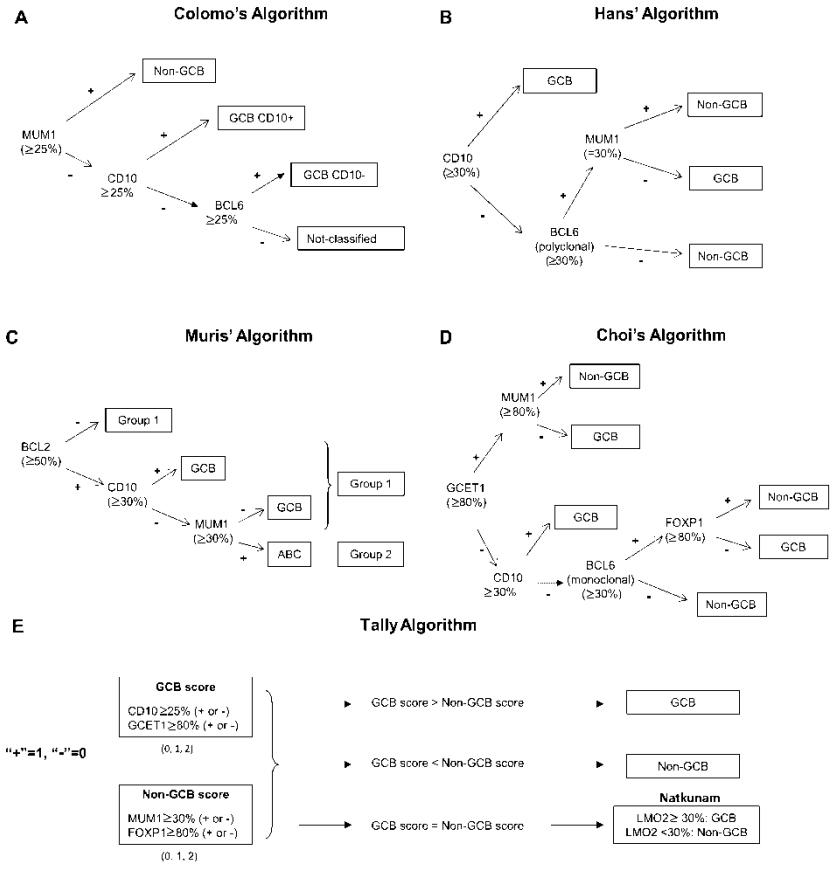

Figure 22. Five immunostaining algorithms to assess differentiation profile (germinal center [GCB] *vs.* non-GCB) in patients with diffuse large B-cell lymphoma.

Gene expression profiling (GEP)

A subset of 62 samples with RNA extracted from fresh frozen lymph node was investigated using Affymetrix HG U133 plus 2.0 gene expression arrays. All gene expression array data were normalized using MAS5.0 software, and were log2 transformed. We used the bayesian compound covariate predictor of the ABC/GCB DLBCL previously described.(49, 124) All samples predicted as ABC DLBCL with greater than 90% were called ABC DLBCL. The samples that showed less than 10% of probability of being call ABC DLBCL were classified as GCB DLBCL. All the other cases were considered unclassified DLBCL.

Statistical análisis

The definitions of CR, PFS and OS were the standard.(116) Categorical data were compared using Fisher's exact test, two-sided *P* value, whereas for ordinal data non-parametric tests were used. The multivariate analysis of the variables predicting response was performed by logistic regression method.(125) The actuarial survival analysis was performed according to Kaplan and Meier method and the curves compared by the log-rank test.(126) The multivariate analysis for survival was performed using stepwise proportional hazards model (Cox).(107)

Results

Immunophenotypic profile

The immunohistochemistry expression of single antigens is detailed in Table 10. The distribution of the patients according to the differentiation phenotype (GCB *vs.* non-GCB) after applying the different algorithms is shown in Table 11. The distribution of the cases according to the phenotype was similar for Colomo and Hans' algorithms. In the Choi and Tally algorithms the proportion of non-GCB cases assigned was significantly higher (non-GCB *vs.* GCB, 67% *vs.* 33% and 63% vs. 37%, respectively). In the Muris' algorithm, the majority of the cases were allocated as GCB phenotype (GCB *vs.* non-GCB, 57% *vs.* 43%). The Colomo's algorithm is the only, of the 5 algorithms that considers a category of "not classified" and 23 tumors were included in this group. Although, most of these cases would be categorized in the non-GCB subtype in the other classifiers we excluded the Colomo's algorithm for the comparison between the other algorithms. Thus, the other 4 algorithms (Hans, Muris, Choi and Tally) were completely assessed in 135 patients, with 111 cases (82%) being allocated in the same GCB or non-GCB group.

Gene expression profiling: concordance with immuno-histochemistry

GEP data were available in 62 patients: 30 cases were allocated to GCB origin (48%) and 22 cases to ABC origin (36%), whereas 10 cases (16%) were considered unclassified. No differences in terms of initial features and outcome were observed between the group with GEP information and without (data not shown).

Antigen	*No. of assessable samples*	*No.of positive (%)*
CD10*	157	41 (26)
BCL6*	146	94 (64)
GCET1 *	146	67 (46)
HGAL	139	61 (44)
IRF8	147	42 (29)
LMO2*	143	60 (42)
JAW1	149	126 (85)
MUM1/IRF4*	148	41 (28)
FOXP1*	143	112 (78)
BLIMP1	147	66 (45)
XBP1	104	28 (27)
BCL2*	152	76 (50)

Table 10. Immunohistochemical expression of single antigens in 157 patients. *Antigens used to build up the algorithms

The relationship between the expression of single antigens and GEP is shown in Table 12. Only CD10 and JAW1 expression significantly correlated with a GCB origin according to GEP (p=0.007 and p=0.01, respectively), whereas MUM1/IRF4 expression correlated with ABC origin (p=0.02). The concordance between the GEP profiles and the patterns of immunohistochemistry as assessed by the five algorithms is shown in Table 13. A significant correlation was observed between GEP data and Colomo (p=0.02), Hans (p=0.015), Muris (p=0.04) and Tally (p=0.02), whereas the correlation did not reach significant value with Choi (P=0.1). A higher percentage of misclassified cases in the GCB-phenotype subset than in the non-GCB subgroup was observed. The proportion of GCB-

DLBCL cases that were not correctly allocated by immunohistochemistry was of 41%, 48%, 30%, 60% and 46% for Colomo, Hans, Muris, Choi and Tally, respectively.

Algorithm	*N (%)*	*CR rate N (%)*	*5-year PFS (%)*	*5-year OS (%)*
Colomo				
GCB	53 (44)	39 (74)	48	54
Non-GCB	68 (56)	53 (78)	55	62
Hans				
GCB	61 (41)	47 (77)	54	60
Non-GCB	88 (59)	67 (76)	52	59
Muris				
GCB	87 (57)	63 (72)	48	57
Non-GCB	65 (43)	51 (78)	56	63
Choi				
GCB	45 (33)	32 (71)	48	54
Non-GCB	90 (67)	70 (78)	52	61
Tally				
GCB	55 (37)	45 (82)	63	56
Non-GCB	92 (63)	65 (71)	54	47

Table 11. Distribution, complete response [CR], progression-free survival [PFS] and overall survival [OS] of 157 patients with diffuse large B-cell lymphoma according to the immunophenotype (germinal-center [GCB] *vs.* non-GCB)

On the other hand, the proportion of ABC patients that were not properly assigned according to the different algorithms (Colomo, Hans, Muris, Choi and Tally) was 19%, 15%, 38%, 16% and 20%, respectively. The sensitivity in the GCB group was 59%, 52%, 70%, 40% and 53% for Colomo, Hans, Muris, Choi and Tally algorithms, respectively. The sensitivity in the non-GCB group was 81%, 85%, 62%, 84% and 80%, respectively.

		GEP		
Antigen		***No. assessable patients***	***Germinal center N (%)***	***Activated (ABC) N (%)***
CD10*	(-)	52	19 (47)	21 (53)
	(+)		11 (92)	1 (8)
BCL6	(-)	48	12 (44)	15 (56)
	(+)		15 (71)	6 (36)
MUM1**	(-)	49	22 (68)	10 (32)
	(+)		6 (35)	11 (65)
IRF8	(-)	50	21 (64)	12 (34)
	(+)		7 (41)	10 (59)
GCET1	(-)	48	12 (52)	11 (48)
	(+)		16 (64)	9 (36)
GCET2	(-)	50	12 (55)	10 (45)
	(+)		16 (57)	12 (43)
FOXP1	(-)	49	6 (86)	1 (14)
	(+)		21 (50)	21 (50)
LMO2	(-)	48	10 (48)	11 (52)
	(+)		18 (67)	9 (33)
JAW1***	(-)	50	0 (0)	4 (100)
	(+)		29 (63)	17 (37)
BLIMP1	(-)	49	14 (54)	12 (46)
	(+)		15 (65)	8 (35)
XBP1	(-)	25	10 (63)	6 (37)
	(+)		7 (78)	2 (22)
BCL2	(-)	51	20 (67)	10 (33)
	(+)		9 (43)	12 (21)

Table 12. Correlation between gene expression profiles (GEP) and different single antigens in 52 DLBCL patients with available microarrays data. *P=0.007. **P=0.01. ***P=0.02

Clinical significance of GEP and immunohistochemistry profiles

CR rate of the series was 76%. CR achievement was observed more frequently in patients with absence of B-symptoms, ambulatory performance status, absence of extranodal involvement or bulky mass, normal serum β2-m, adriamycin-containing chemotherapy and low-risk IPI. Neither single antigen

expression nor the differentiation profiles as assessed by the immunohistochemistry algorithms were able to predict CR (Table 11). GEP profiles did not predict CR.

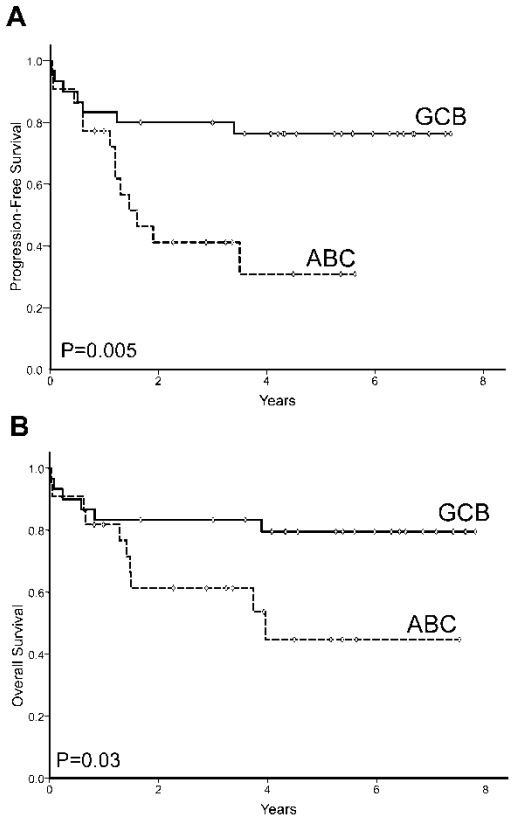

Figure 23. Progression-free survival (PFS) and overall survival (OS) of 52 patients with gene expression profile data available according to the molecular subtype (germinal center B-cell-like (GCB) vs. activated B-cell-like (ABC) profile).

Seventy four of 157 patients eventually progressed, including 36 of the 119 patients who reached a CR. The 5-year PFS was 50% (95% CI: 42-58%) (Figure 21). Variables with unfavorable prognostic value for PFS were: presence of B-symptoms, non-ambulatory performance status (ECOG≥2), extranodal involvement ≥1 sites, advanced Ann Arbor stage (III-IV), high

serum LDH, high serum β2-m levels and high risk IPI. High BCL2 protein expression also predicted poor PFS (5-year PFS 27% *vs.* 56% for BCL2 >75% *vs.* ≤75%, respectively; *P*=0.003). In addition, the differentiation profile (GCB *vs.* ABC) as assessed by GEP was able to predict PFS (5-year PFS, 76% *vs.* 31%, respectively; P=0.005) as shown in Figure 23A. On the contrary, the immunophenotypic profiles as assessed by the five different algorithms did not show significant prognostic value for PFS (Table 11). Sixty-one patients died during follow-up, with a 5-year OS of 58% (95% CI: 50–66%) (Figure 21). Unfavorable variables predicting OS were: age >60 years, poor performance status (ECOG≥2), advanced Ann Arbor stage (III-IV), high serum LDH levels, high serum β2-m level and high-risk IPI.

Patients treated with adriamycin-containing chemotherapy also showed a significant advantage in terms of OS. Among the single antigens tested, only BCL2 overexpression (>75%) correlated with poor OS. Differentiation profile as assessed by GEP (GCB *vs.* ABC) showed a significant prognostic value for OS in the subset of patients with this GEP information, with a 5-year OS of 80% *vs.* 45%, respectively; P=0.03) (Figure 23B). By contrast, none of the profiles assessed by immunostaining were able to predict OS as shown in Table 11 and Figure 24. Regarding Muris' algorithm, when using the step 2-algorithm that included BCL2 immunostaining, the algorithm was also unable to predict OS (5-year OS 59% vs. 48%, for groups 1 and 2, respectively; p=0.2). The results were the same when the study was performed in 133 patients receiving strictly R-CHOP (Supplementary Figures S1 and S2).

	GEP	
Algorithm	***Germinal center N (%)***	***ABC N (%)***
Colomo*		
GCB	13 (59)	3 (19)
Non-GCB	9 (41)	13 (81)
Hans**		
GCB	15 (52)	3 (15)
Non-GCB	14 (48)	17 (85)
Muris***		
GCB	21 (70)	8 (38)
Non-GCB	9 (30)	13 (62)
Choi****		
GCB	10 (40)	3 (16)
Non-GCB	15 (60)	16 (84)
Tally*****		
GCB	15 (54)	4 (20)
Non-GCB	13 (46)	16 (80)

Table 13.Concordance between the gene expression profile (GEP) (Germinal center (GCB) *vs.* activated (ABC)) and the immunohistochemistry patterns (GCB *vs.* non-GCB) as assessed by five algorithms in 52 patients with available GEP information.

A multivariate analysis was performed in the 52 patients with GEP information including differentiation profile (GCB *vs.* ABC profile), BCL2 expression (≤75% vs. >75%) and IPI. In the final model with 52 cases, differentiation profile was the most important variable to predict OS (RR: 3.3; p=0.04).

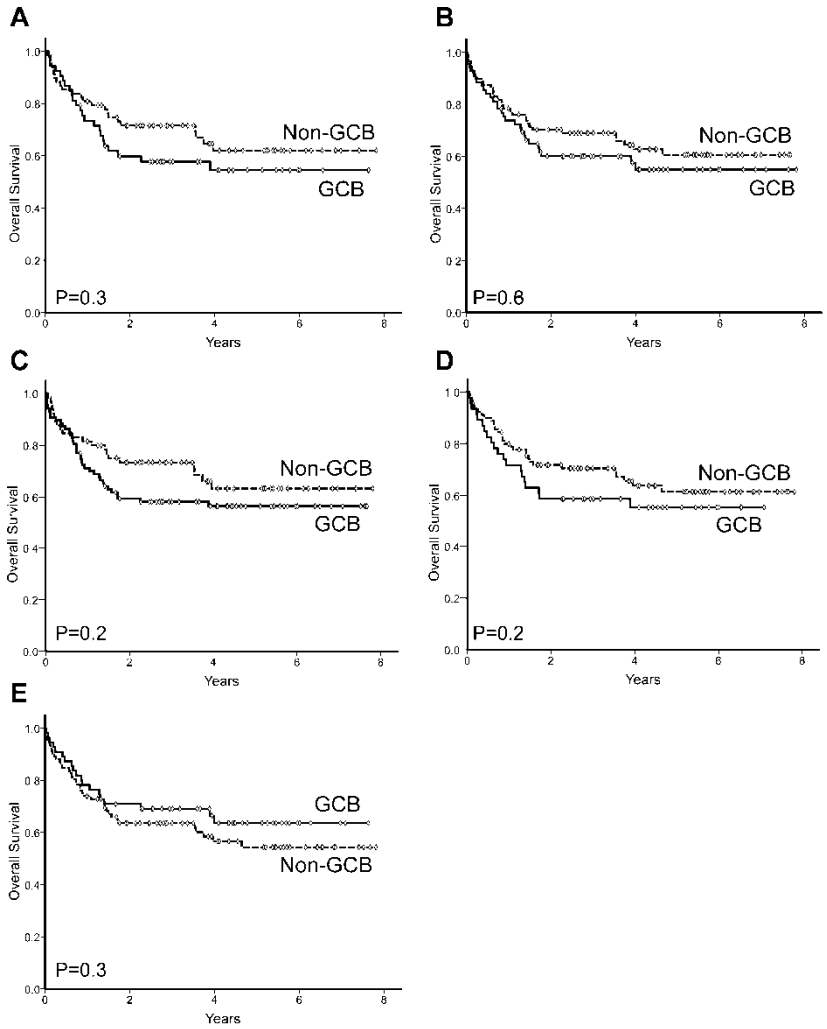

Figure 24. Overall survival of 157 patients with diffuse large B-cell lymphoma according to the differentiation profile (Germinal center [GCB] *vs.* non-GCB) as assessed by five immunohistochemistry algorithms: A) Colomo's algorithm, B) Hans' algorithm, C) Muris' algorithm, D) Choi's algorithm and E) Tally algorithm.

Discussion

Two types of DLBCL can be recognized on the basis of GEP data: GCB-DLBCL and ABC-DLBCL. These groups have a different cell origin and a distinct clinical behavior, with patients carrying an ABC-DLBCL showing a poorer outcome than patients with a GCB tumor.(75) This fact, originally described in the pre-rituximab era, has been recently confirmed in patients receiving immunochemotherapy.(49) In addition to the prognostic interest, the molecular classification might be useful to select treatments that could be active only in specific subtypes of DLBCL, such as the effective use of bortezomib in ABC-DLBCLs.(67, 127, 128) Thus, GEP information is not only of academic interest, but it could be an important decision element in the management of the patients in the near future. Unfortunately, the GEP assessment using microarrays is not feasible in the routine clinical practice. For this reason different attempts have been made to capture the prognostic categorization of GEP information using a more friendly technical approach such as immunohistochemistry. This method can be performed in archival formalin-fixed paraffin-embedded tissues and is available at any laboratory of Pathology. Different algorithms have been created combining the expression of well-known antigens, including the ones analyzed in the present study (Colomo, Hans, Muris, Choi and Tally; Figure 22).(36, 47, 48, 79, 80) The real value of these algorithms as a surrogate of GEP data and to define the prognosis of patients with DLBCL is a subject of controversy and it constitutes the aim of the present study. This study was performed in a homogeneous and

representative series of patients with DLBCL diagnosed and treated with immunochemotherapy in five hospitals from the GELCAB. Clinical features and outcome are the expected for an unselected series of DLBCLs. TMA construction and the assessment of the different antigens were performed based on information from original descriptions.(36, 47, 48, 79, 80) Although a significant association between GEP and immunophenotypic profiles was observed (table 6), the positive and negative predictive values were poor for the five algorithms tested. In fact, 30-50% of GCB-DLBCLs and 15-25% of ABC-DLBCLs were incorrectly allocated by immunohistochemistry, making its use difficult in the clinical practice. Furthermore, in prognostic terms, the applicability of the algorithms was poor in the current series. Although, the number of cases studied by GEP was relative low, the molecular GCB and ABC subtypes showed a clearly differentiated outcome in terms of PFS and OS. As reflected in Figure 24, none of the five algorithms were capable of defining groups with prognostic impact.

The current results are in contradiction with other studies supporting an excellent correlation between inmunohistochemistry and GEP in terms of prognosis.(47, 48, 79) However, some others studies agree with ours in regard to the lack of clinical significance of the immunophenotypic profiles in patients treated with immunochemotherapy.(37) Several reasons may account for these discrepancies, including the population studied and the methodology used. First, retrospective analysis of heterogeneous series with different therapies may have confounded the results. In this respect, it has to be noted that the present is rather a population-based series, not biased, with

patients homogenously treated with immunochemotherapy. On the other hand, we have included both nodal and extranodal DLBCLs. The main clinical features, including IPI, seem to be very similar to other publications.(47) Secondly, technical shortcomings and inter-observer discrepancies in the interpretations of the immunostaining slides most likely have an important role in the divergent data. The liability of immunohistochemistry has been pointed out in several papers, including the Lunenburg Consortium study that was an important step forward to standardize the immunohistochemical studies in these lymphomas.(117) In our study we followed the Lunenburg indications to evaluate the antigens described in the consortium. However, some of the antigens used to build up the algorithms are relatively new and the evaluation criteria have not been so thoroughly investigated. Therefore, we cannot rule out that differences in the evaluation of the immunostaining with the newer markers could be part of the discrepant results with the more recent described algorithms. In this sense, it is of note the low positive value of markers considered absolutely typical of GCB (i.e. GCET1 and LMO2 were positive in 64% and 67% of the GCB cases, respectively) or ABC subtype (i.e., FOXP1 was positive in 50% of the ABC cases) as shown in table 5. However, we observed also discrepant results using the more established algorithms suggesting that the reasons for the discordances may not be only attributed to interpretative differences. We believe that one of the most important difficulties in the immunohistochemical attempts to reproduce the GEP results is the limitation to capture the information obtained from complex gene expression signatures on a large number of genes using a

very small number of antigens. Thus, to reliably reproduce the GEP information and to transfer it to the clinical practice, new technologies, probably different from the standard inmmunohistochemistry, are warranted.

In summary, in the present study none of the five immunohistochemical algorithms were able to accurately predict the GEP subtype or to separate molecular groups with prognostic value. As a consequence, stratification based on immunohistochemical algorithms for guiding therapy should be viewed very cautiously, even in clinical trials.

Supplementary information

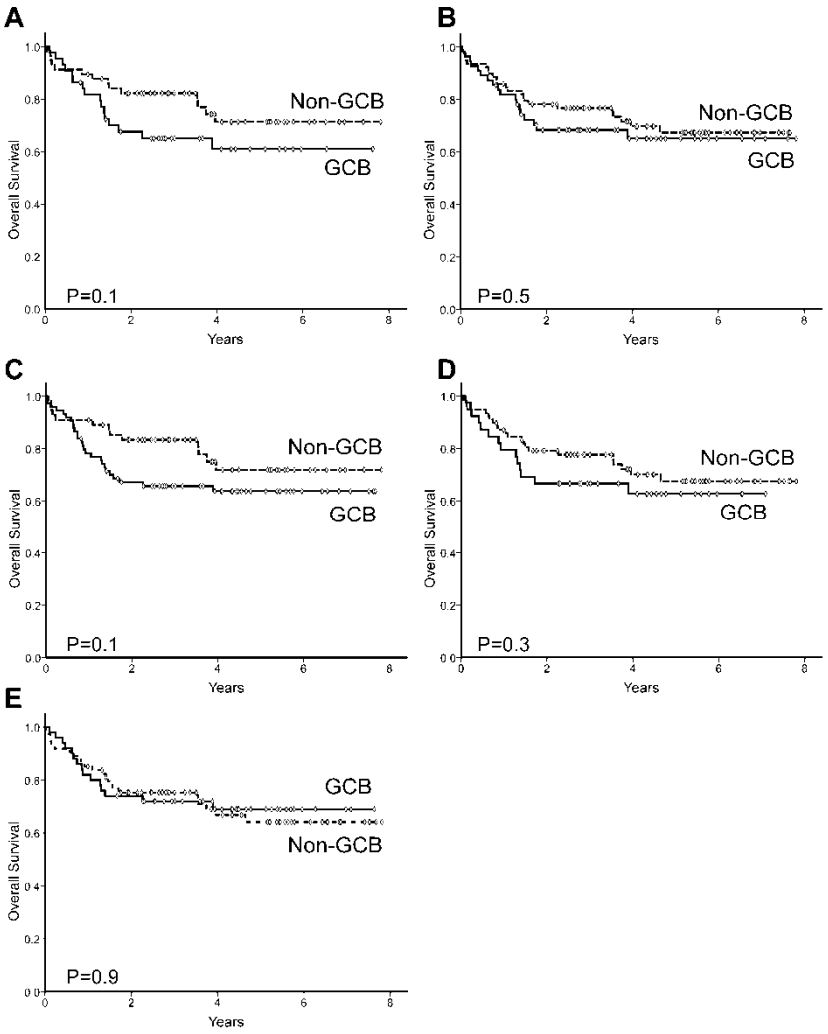

Figure S1. Overall survival of the 133 patients strictly receiving R-CHOP according to the different algorithms (A. Hans; B. Colomo; C. Muris; D. Choi; E. Tally).

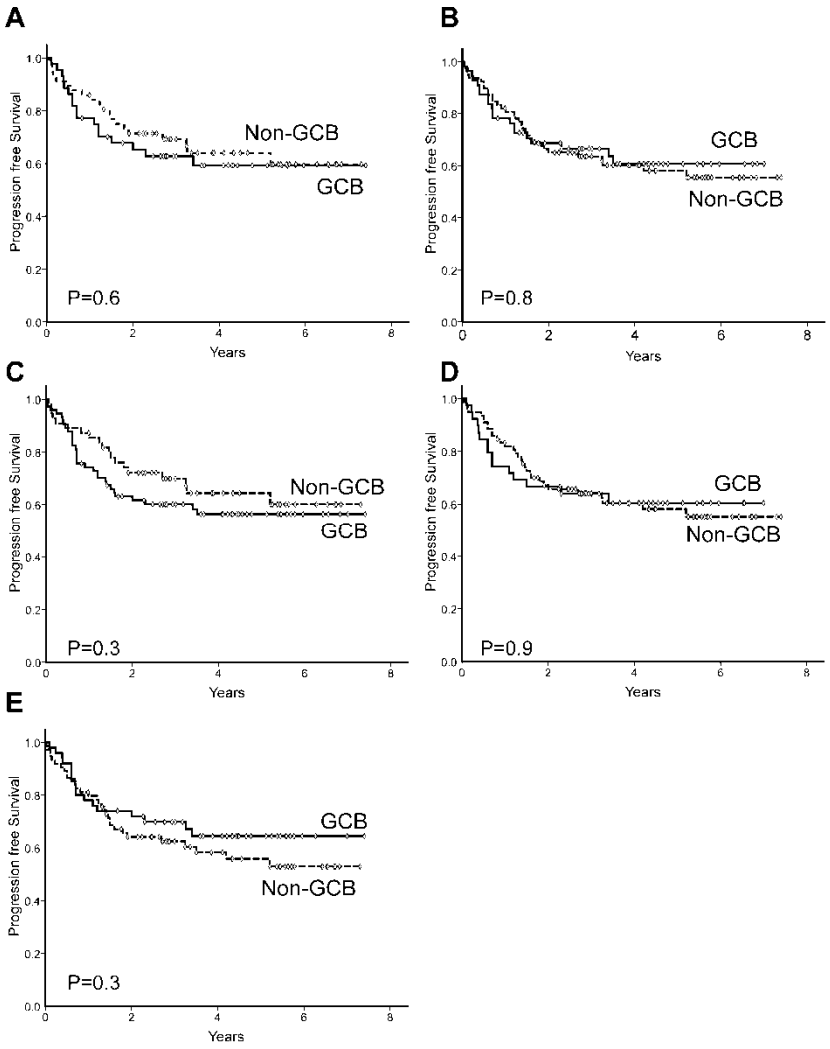

Figure S2. Progression-free survival of the 133 patients strictly receiving R-CHOP according to the different algorithms (A. Hans; B. Colomo; C. Muris; D. Choi; E. Tally).

IV. DISCUSION

Discusión

1. Discusión

Los trabajos que componen esta tesis han permitido profundizar un poco más en la caracterización biológica, clínica y pronostica del LDCGB. La base para ambos estudios se fundamenta en la disponibilidad de series importantes de pacientes con LDCGB diagnosticados y tratados de manera homogénea. En el primer trabajo, los pacientes provienen de un solo centro (Hospital Clínic de Barcelona), mientras que en el segundo, corresponden a una serie de pacientes del "*Grup per l'estudi dels Limfomes de Catalunya I Balears"(GELCAB)* formado por la mayoría de Servicios de Hematología de Cataluña y Baleares. El diagnostico, tratamiento y seguimiento de los pacientes ha sido homogéneo, gracias a seguir protocolos comunes. En los dos trabajos ha sido primordial la identificación y revisión histológica de los casos, la construcción de plataformas de perfiles de expresión génica y de tejido, así como el uso de instrumentos estadísticos para configurar los resultados.

El primer intento de caracterizar de forma integral el LDCGB surgió con el desarrollo de la clasificación REAL. La clasificación actual de las neoplasias linfoides supone una

integración de los diferentes aspectos biológicos (morfología, inmunofenotipo, citogenética y biología molecular), clínicos (formas de presentación y origen primario) y pronósticos. Todo ello dirigido a establecer categorías nosológicas con personalidad específica. En este marco, el LDCGB es una categoría que a priori parece bastante homogénea dado el punto de vista morfológico, inmunofenotípico y clínico. Sin embargo, al profundizar, se evidencia que ello no es así. En efecto, existe heterogeneidad morfológica (formas centroblásticas, inmunoblásticas y anaplásicas), inmunofenotípicas y moleculares. Dado el punto de vista clínico, el LDCGB de origen primario extraganglionar parece intrínsecamente diferente del ganglionar, al menos para determinadas localizaciones. Más recientemente se han diferenciado unos grupos moleculares que se discutirán más adelante. Todos estos aspectos no permiten diferenciar grupos distintos en la clasificación de la OMS, básicamente debido al hecho que no existen categorías claras y reproducibles para definirlos.

El primer trabajo ha contribuido a estudiar los LDCGB de origen primario extraganglionar del LDCGB como entidades con características diferentes de las formas ganglionares. Así estos

pacientes presentan con mayor frecuencia características pronosticas favorables al diagnóstico (estadio temprano, niveles séricos bajos de LDH, e IPI de bajo riesgo), así como en la evolución (tasa de RC, SLP y SG superior). Sin duda el origen centrogerminal del tumor tiene mucho que ver con la evolución. En el aspecto terapéutico, el impacto pronóstico favorable que ha generado la introducción de la inmunoquimioterapia con rituximab, ha sido el mayor avance en el tratamiento del LDCGB durante la última década. De manera interesante, el valor de los factores pronósticos, tanto clínicos como biológicos, ha cambiado sustancialmente con la inmunoterapia. Por un lado, algunos de los factores pronósticos, como el nuevo IPI, han sido validados en series homogéneas de pacientes tratados con R-CHOP. Por el contrario, otros factores como la expresión de BCL6 probablemente han perdido valor al revaluarse en la era de la inmunoquimioterapia. Hoy en día, sabemos que el impacto desfavorable observado en los pacientes con LDCGB BCL6 (-) ha sido contrarrestado por la inmunoquimioterapia. En cuanto, al claro papel pronóstico adverso de la expresión de BCL2 en la etapa pre-R, en la era del rituximab es controvertido. En este contexto, la segunda contribución de esta tesis doctoral yace en cuestionar el papel de la inmunoquimioterapia en el tratamiento

de los LDCGB extraganglionares. En esta entidad, quizás por las características iniciales de buen pronóstico, no observamos un incremento significativo de la supervivencia en aquellos pacientes tratados con R-CHOP. Ello podría suponer una ausencia de beneficio en estos pacientes, o simplemente que el número de pacientes para las diferentes localizaciones extraganglionares no es suficiente para detectar las diferencias que existen. En cualquier caso, esta observación deberá ser comprobada en estudios prospectivos, aspecto este que resulta probablemente poco realista.

El LDCGB-NOS puede ser separado en subgrupos morfológicos, inmunofenotípicos y moleculares (Tabla 4), la dudosa reproducibilidad de la morfología ha cuestionado su validez. En concreto, el valor pronóstico adverso de la variante morfológica inmunoblástica recientemente confirmado es incierto. El inmunofenotipo no ayuda de manera importante en la subclasificación. Por otro lado, los subtipos de LDCGB establecidos a partir de los estudios de expresión genotípica (CGB y ABC) son los únicos que han podido ser reproducidos en algunas series independientes. Más importante aún, es el impacto pronóstico desfavorable observado en el subgrupo ABC. Sin

embargo, la imposibilidad en la implementación rutinaria de los GEP es el principal factor limitante para el uso extendido de esta técnica. La inmunohistoquímica intenta de alguna manera remedar la información obtenida con los perfiles de expresión genotípica. Así, contamos con diversos algoritmos (Figuras 13-17) que utilizan la expresión individual de antígenos relacionados con la diferenciación centrogerminal y post-centrogerminal. Estos algoritmos permiten realizar una categorización inmunofenotípica (CGB y no-CGB). No obstante, el inmunofenotipo presenta dos grandes problemas que pueden explicar los resultados no concordantes: 1) problemas de estandarización, y 2) la información basada en 2-5 antígenos es difícil que remede los datos de varios cientos de genes. En este sentido, esta tesis permite confirmar el valor pronóstico de los estudios de expresión genotípica en el linfoma difuso de células grandes en pacientes tratados con inmunoquimioterapia con rituximab. (Figura 23) Sin embargo, nuestros resultados cuestionan el valor de los estudios de inmunohistoquímica como remedo de los perfiles de expresión genotípica. Aunque se encontró una correlación significativa entre los GEP y los diferentes algoritmos inmunohistoquímicos (Tabla 13), los valores predictivo negativo y positivo fueron relativamente bajos para los cinco algoritmos

utilizados. De hecho, 30-50% de los LDCGB de origen CGB y 15-25% de los ABC fueron incorrectamente localizados por la inmunohistoquímica. En términos de supervivencia global, como se puede observar en la Figura 24, ninguno de los 5 algoritmos fue capaz de definir grupos con impacto en el pronóstico.

Estos dos trabajos definen dos puntos importantes en el LDCGB. En primer lugar, establecen una base sólida que soporta al LDCGB-EG como entidad propia con comportamiento clínico particular y menor impacto en la supervivencia en la era de la inmunoquimioterapia.

Como segundo aspecto de importancia, esta tesis confirma el valor pronóstico de los subgrupos moleculares (CGB y ABC) del LDCGB, pero no el de los grupos basados en la inmunohistoquímica. Como conclusión final, la utilización de la inmunohistoquímica para definir grupos pronósticos y determinar el tratamiento de los pacientes debe ser considerado con cautela.

V. CONCLUSIONES

Conclusiones

1. Conclusiones

1. Los linfomas de origen primario extraganglionar presentan características clínicas favorables y mejor pronóstico que las formas ganglionares.

2. La adición de rituximab a la quimioterapia convencional ha mejorado sustancialmente la respuesta al tratamiento y la supervivencia de los pacientes con LDCGB tratados con intención curativa.

3. Sin embargo, en el grupo de linfomas de origen primario extraganglionar no se ha observado ese mismo incremento significativo de respuesta y supervivencia.

4. Estas observaciones deben ser confirmadas en estudios prospectivos.

5. Los estudios de expresión genotípica (GEP) en el LDCGB permiten definir dos grupos de pacientes (CG y ABC) con características claramente diferenciadas.

6. En pacientes tratados con inmunoquimioterapia se confirma el valor pronóstico del grupo molecular del LDCGB (CG frente a ABC).

7. Ninguno de los cinco algoritmos inmunohistoquímicos estudiados permite remedar la información obtenida con GEP.

Conclusiones

8. Los grupos moleculares definidos mediante los algoritmos inmunohistoquímicos (CG frente a no-CG) no tienen impacto pronóstico en pacientes con LDCGB tratados con inmunoquimioterapia.

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VII. ANEXOS

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ORIGINAL ARTICLE: CLINICAL

Clinico-biological characterization and outcome of primary nodal and extranodal diffuse large B-cell lymphoma in the rituximab era

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Abstract

To study the main clinico-biological characteristics and the outcome of patients with diffuse large B-cell lymphoma (DLBCL) according to the primary site (nodal vs. extranodal), we included 262 patients consecutively diagnosed with DLBCL in a single institution, 5 years before and after immunochemotherapy was considered as the standard treatment. Altogether 116 patients received CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) and 146 rituximab plus CHOP (R-CHOP). The primary site was the lymph node in 140 patients (53%), Waldeyer's ring (WR) in 22, gastrointestinal (GI) in 33, and other extranodal in 67. The addition of rituximab significantly improved the CR rate in nodal, but not in extranodal, lymphomas. Patients receiving R-CHOP showed higher OS than those treated with CHOP alone (5-year OS: 71% vs. 48%). This difference was maintained in primary nodal (5-year OS: 69% vs. 37%, $p < 0.0001$), but was not observed in primary extranodal (75% vs. 65%, $p = 0.45$) lymphomas. The IPI, treatment, and primary site were the main variables for OS in multivariate analysis. In nodal cases, IPI and treatment maintained value, whereas only IPI predicted OS in extranodal cases. In conclusion, immunochemotherapy treatment dramatically improved the outcome of patients with nodal DLBCL; however, its effect was less in primary extranodal cases, so the prognosis of patients with nodal and extranodal lymphomas has been equalized in the rituximab era.

Keywords: *Diffuse large B-cell lymphoma, extranodal, immunophenotyping profile, rituximab, immunochemotherapy, prognosis*

Introduction

Around one-third of non-Hodgkin lymphomas arise in tissues other than lymph nodes, usually being termed extranodal lymphomas [1–4]. The incidence of lymphomas has increased during recent decades, with that of extranodal lymphomas increasing more rapidly than nodal [3,5]. Although there are multiple publications in the literature dealing with etiopathogenesis, biological features, clinical characteristics, and outcome of extranodal lymphomas, most of them include heterogeneous series of patients, in which several histologic subgroups are usually merged [3,4,6–13]. Diffuse large B-cell lymphoma (DLBCL) is the commonest type of non-Hodgkin lymphoma in Western countries, representing around 30% of them [14]. About one-third of DLBCLs have a primary extranodal origin. Although considered a single category in the Revised European-American Lymphoma/World Health Organization (REAL/WHO) classification, DLBCL most likely includes different clinicopathologic entities that are currently

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difficult to separate with the standard techniques [1,15–17]. In this setting, extranodal DLBCL might also have a specific biologic behavior [18].

Since the incorporation of rituximab (an anti-CD20 chimeric monoclonal antibody) into the armamentarium against lymphomas, the combination of chemotherapy plus rituximab, so-called immunochemotherapy, has become the gold-standard treatment in DLBCLs [19]. Thus, up to 15–20% of improvement in overall survival (OS) has been demonstrated in different settings of patients with respect to chemotherapy alone [20–24]. Nevertheless, the effectiveness of immunochemotherapy has been questioned in some cases, such as in patients with no tumor expression of bcl-2, those with bcl-6 expression, or those of germinal-center origin [25–32]. Little information is available on the efficacy of immunochemotherapy in patients with extranodal DLBCL in large series of patients, although immunochemotherapy is considered the standard of care.

The aim of the present study was to investigate the main clinico-biological features of patients with DLBCL according to the primary origin of the disease (nodal vs. extranodal), as well as to analyze the impact of the addition of rituximab to the standard CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) regimen in these groups of patients.

Patients and methods

Patients

Three hundred sixty-eight patients consecutively diagnosed with a CD20-positive DLBCL between January 1997 and December 2006, and followed up in a single institution, were selected for the present study. The cases with recognized disease phase of a follicular lymphoma or another type of indolent lymphoma with subsequent transformation into a DLBCL, primary mediastinal, intravascular, and primary effusion were not included. In addition, immunodeficiency associated tumors (patients human immunodeficiency virus [HIV]-positive, 41 cases; post-transplant lymphoproliferative disorders, five cases) and primary central nervous system (CNS) lymphoma (12 cases) were excluded from the study. Finally, 48 patients who received non-adriamycin-containing chemotherapy for different reasons were also excluded. Thus, the remaining 262 patients constituted the subjects of the present study.

Median age of the patients was 60 years (range, 19–81) and the male/female distribution was 134/128. Main initial characteristics of the patients are listed in Table I. Advanced stage (Ann Arbor III or IV) was observed in 140 cases (55%), and any extranodal involvement in 181 (69%), including bone marrow infiltration in 52 cases (20%). Altogether, 133 patients of 244 with available data (55%) had high serum lactic acid dehydrogenase (LDH) levels, whereas the distribution according to the International Prognostic Index (IPI) was the following: low-risk, 90 cases (37%); low/intermediate, 41 cases (17%); high/intermediate, 51 cases (21%); high-risk, 62 cases (25%); and non-assessable, 18 cases. The main initial and evolutive variables, including the histologic parameters indicated below, were recorded and analyzed for prognosis.

Staging maneuvers included patient history and physical examination (including Waldeyer's ring

Table I. Main clinical features at diagnosis of 262 patients with diffuse large B-cell lymphoma according to the primary site of the disease.

	Nodal ($n=140$)	Waldeyer's ring ($n=22$)	Gastrointestinal ($n=33$)	Other extranodal ($n=67$)
Gender (M/F)	67/73	12/10	19/14	36/31
Age (median; range)	60 (19–81)	65 (26–80)	54 (24–78)	60 (21–81)
B-symptoms (%)	41	9*	28**	34
ECOG $\geq$2 (%)	46	9*	31	45
Advanced stage (%)	61	23*	30**	64
Bone marrow (+) (%)	26	9*	0**	22
High serum LDH (%)	59	43	33**	59
High serum β_2m (%)	55	15*	33	49
IPI (risk)				
Low (%)	30	71	58	30
Low/intermediate (%)	20	0	14	17
High/intermediate (%)	26	24	7	15
High (%)	24	5*	21**	38

*$p < 0.01$ vs. other groups; **$p < 0.05$ vs. other groups. The number of patients with available data for ECOG, bone marrow, LDH, β_2m, and IPI were 255, 260, 244, 216, and 244, respectively.

ECOG, Eastern Cooperative Oncology Group; LDH, lactic acid dehydrogenase; β_2m, β_2-microglobulin; IPI, International Prognostic Index.

[WR] area), blood cell counts, and serum biochemistry, including LDH and β_2-microglobulin (β_2m) levels, computed tomography scans of chest, abdomen, and pelvis, as well as bone marrow biopsy.

Treatment

The period of time of the study was 5 years before (1997–2001) and after (2002–2006) rituximab-containing chemotherapies were established as the standard treatment for patients with DLBCL. Thus, before January 2002, 116 patients (44%) received the CHOP regimen, and after that time, 146 (56%) patients were treated with CHOP plus rituximab (R-CHOP). The distinction between nodal and extranodal was not taken into account to plan the treatment. No significant differences were observed in the main initial features of the patients according to the treatment given (CHOP vs. R-CHOP) (data not shown), and, in addition, the policy for using prophylactic antibiotic and granulocyte colony-stimulating factor (G-CSF) did not vary over time.

Post-therapy re-staging consisted of repetition of the previously abnormal tests and/or biopsies. Response was assessed according to conventional criteria [33]. Overall, 177 patients achieved a complete response (CR) (69%), 28 patients a partial response (11%), and 50 (20%) patients failed to respond to treatment. In seven cases the response was not assessable. After a median follow-up of 4.9 years (range, 0.2–11.9) for surviving patients, 112 patients have died. The 5-year and 10-year overall survival was 60% (95% confidence interval [CI]: 54–66%) and 53% (95% CI: 45–61%), respectively [Figure 1(A)]. Median follow-up for patients receiving CHOP and R-CHOP was 8.5 and 3.7 years, respectively.

Nodal/extranodal definitions

Lymphomas arising in extranodal organs, with no or only minor lymph node involvement, were considered as primary extranodal. Lymphomas with lymph node involvement clinically dominant, as well as those presenting at the spleen, were considered as primary nodal. Lymphomas from the Waldeyer's ring, although considered nodal, were analyzed separately. Finally, those lymphomas with extensive disease involving both nodal and extranodal sites were considered as nodal [9,12,18].

Histologic features

The diagnosis of DLBCL was based in all cases on the criteria established in the WHO classification [1]. For the morphologic analysis, all histologic slides were reviewed by three different observers (A.M., L.C., E.C.). The panel of monoclonal antibodies included antibodies against the following antigens: CD20, CD79a, CD3, CD5, CD10, MUM-1/IRF4, CD138, bcl-2, bcl-6, p53, and p27. The proliferative index was assessed by Ki-67 immunostaining. The antibodies and the immunohistochemical conditions of use have been previously described [34]. The patients were assigned to germinal center B-cell-like (GCB) or non-GCB groups according to methods previously described [34,35].

Statistical analysis

Categorical data were compared using Fisher's exact test, two-sided p-value, whereas for ordinal data non-parametric tests were used. Multivariate analysis of the variables predicting response was performed using logistic regression. The definitions of CR,

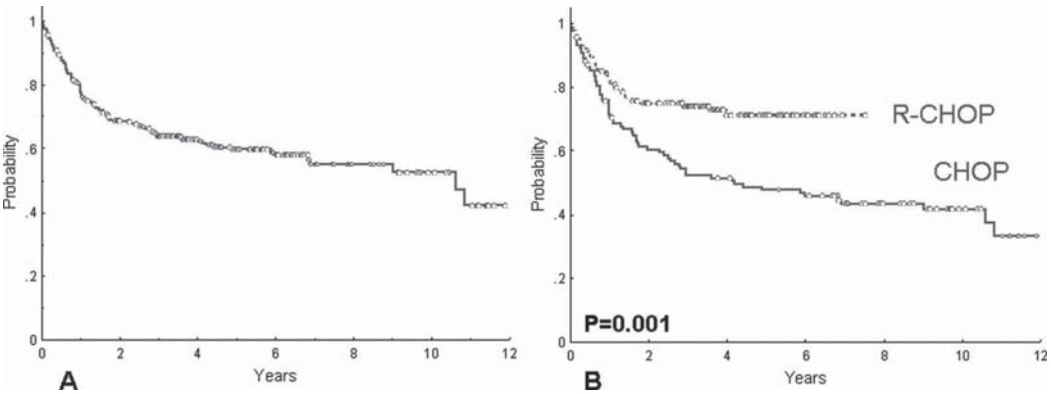

Figure 1. (A) Overall survival (OS) of 262 patients with diffuse large B-cell lymphoma treated with curative intention; (B) OS of the same 262 patients according to the therapy given: CHOP vs. rituximab plus CHOP (R-CHOP).

disease-free survival (DFS), and OS were the standard [36]. Actuarial survival analysis was performed according to the method described by Kaplan and Meier [37], and the curves compared by log-rank test [38]. Multivariate analysis for survival was performed by using the stepwise proportional hazards model (Cox) [39].

Results

Distribution and clinico-biological features

One hundred patients (38%) presented at a primary extranodal site and 22 patients (9%) at WR, whereas the remaining one hundred forty (53%) had a primary nodal DLBCL, including one lymphoma of the spleen. Distribution according to the extranodal site was the following: gastrointestinal (GI) tract (33 cases, 12% of the overall series; with gastric lymphoma found in 30 cases), soft tissue (16 cases; 6%), breast (13 cases; 5%), lung and pleura (11 cases; 4%), liver (nine cases; 3%), bone (nine cases; 3%), kidney (three cases), testis/ovary (two cases), bone marrow (two cases), skin, and thyroid (one case each).

The main clinical features at diagnosis according to the primary site of the lymphoma are detailed in Table I. Patients with WR DLBCL more frequently presented with early stage, ambulatory performance status, absence of B-symptoms, normal serum albumin and β_2m levels, and low- or low/intermediate-risk IPI ($p < 0.01$ in all cases vs. the other groups). GI DLBCL also more frequently had an early stage, absence of bone marrow infiltration, normal serum LDH, and low- or low/intermediate-risk IPI than the other groups ($p < 0.05$ in all cases).

Immunohistochemical expression of the main single antigens according to the origin of the lymphoma is summarized in Table II. No significant differences were found regarding antigen expression or differentiation profile as defined by Hans' algorithm [35].

Response to treatment

No differences were found in the treatment given to the patients (CHOP vs. R-CHOP) according to the primary origin of the lymphoma. One hundred seventy-seven of 255 assessable patients (69%) achieved CR. Patients with lymphoma of WR (19/21, 90%) and the GI tract (29/32, 91%) showed a higher CR rate than the others. Patients receiving R-CHOP showed a higher CR rate than those treated with CHOP (78 vs. 60%, respectively; $p = 0.002$). Moreover, the proportion of primary refractory patients was significantly lower in those treated with R-CHOP than in those receiving CHOP (16 vs. 23%, respectively; $p = 0.002$). In Table III, the differences in CR rate and the proportion of primary refractory patients are detailed for the different nodal and extranodal groups. Whereas in nodal lymphomas the addition of rituximab significantly improved the CR rate and decreased the proportion of refractory patients, no significant difference was found between therapies with CHOP and R-CHOP in primary extranodal lymphomas.

The following variables predicted for CR achievement: age < 60 years, ambulatory performance status (Eastern Cooperative Oncology Group [ECOG] <2), absence of B-symptoms, absence of bulky disease, early Ann Arbor stage, no bone marrow involvement, normal serum albumin, and LDH and β_2m levels, as well as the IPI and treatment with R-CHOP. In logistic regression analysis, IPI ($p < 0.001$; relative risk [RR]: 0.13) and R-CHOP ($p = 0.003$; RR: 2.56) were the most important variables to predict CR achievement. In patients with nodal DLBCL, IPI ($p < 0.001$; RR: 0.32) and R-CHOP ($p = 0.03$; RR: 2.5) maintained the predictive value. In primary extranodal cases only IPI ($p < 0.003$; RR: 0.18) and bulky disease ($p = 0.04$; RR: 0.31) had importance for CR, whereas R-CHOP lost its prognostic value.

No single antigen expression or differentiation profile predicted CR achievement. Both germinal-center

Table II. Immunophenotypic features of patients with diffuse large B-cell lymphoma in whom adequate material was available to assess CD10, bcl-6, MUM-1, and bcl-2 expression, according to the primary site of the disease.

	Nodal	Waldeyer's ring	Gastrointestinal	Other extranodal
bcl-2 expression (%)	37/61 (61%)	10/13 (77%)	5/12 (42%)	19/29 (65%)
CD10+ (%)	20/64 (31%)	1/10 (10%)	4/11 (36%)	8/30 (27%)
bcl-6+ (%)	39/63 (63%)	10/12 (83%)	9/11 (82%)	15/25 (60%)
MUM-1+ (%)	26/44 (59%)	7/9 (78%)	1/4 (25%)	6/15 (40%)
Differentiation profile*				
Germinal-center (%)	22/53 (42%)	3/10 (30%)	4/6 (67%)	9/18 (50%)
Non-germinal-center (%)	31/53 (58%)	7/10 (70%)	2/6 (33%)	9/18 (50%)

*As assessed by Hans' method [35].

Table III. Response to treatment, early death, disease-free survival (DFS), and overall survival (OS) of 262 patients with diffuse large B-cell lymphoma according to the treatment given† for the different primary sites of the disease.

	Nodal ($n=140$)	Waldeyer's ring ($n=22$)	Gastrointestinal ($n=33$)	Other extranodal ($n=67$)
CR rate				
CT (%)	51	100	92	59
R-CT (%)	75*	86	90	72
Primary refractory				
CT (%)	33	0	0	17
R-CT (%)	18*	0	0	16
Early death				
CT (%)	9	0	0	14
R-CT (%)	8	0	0	8
5-year disease-free survival				
CT (%)	60	100	75	76
R-CT (%)	75*	85	76	84
5-year overall survival				
CT (%)	36	100	92	58
R-CT (%)	66*	80	90	67

*$p < 0.05$.
†Chemotherapy (CT) vs. rituximab plus chemotherapy (R-CT).

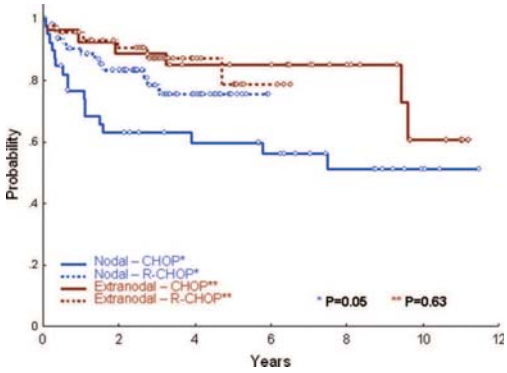

Figure 2. Disease-free survival of patients with diffuse large B-cell lymphoma according to the primary site of the disease (nodal vs. extranodal) and the therapy given (CHOP vs. rituximab plus CHOP [R-CHOP]).

and non-germinal-center lymphomas benefited from the addition of rituximab, irrespective of the nodal or extranodal primary site.

Disease-free survival

Forty-two of 177 CR patients eventually relapsed, with a 5-year DFS of 75% (95% CI: 68–82%). The DFS according to the primary site of the lymphomas is detailed in Table III. Patients with primary extranodal lymphoma showed a higher DFS than those with nodal disease (5-year DFS: 83 vs. 69%, respectively; $p = 0.02$). Overall, patients receiving R-CHOP did not have a significantly longer DFS than those treated with CHOP alone (5-year DFS: 76 vs. 70%, respectively; $p = 0.63$). However, when analyzing by subgroups, as indicated in Table III, primary nodal lymphomas treated with R-CHOP showed a significantly higher DFS than those treated with CHOP alone (5-year DFS: 75 vs. 60%, respectively; $p = 0.05$). DFS curves are depicted in Figure 2.

Overall survival

One hundred and five patients have died during the follow-up. Twenty-seven patients died within 4 months from diagnosis and were considered as 'early deaths.' Treatment with R-CHOP did not influence early deaths either in the whole series or in the primary nodal or extranodal subsets. Five-year OS of the entire series was 60% (95% CI: 54–66%). Unfavorable variables predicting OS were: age >60 years, presence of B-symptoms, poor performance status (ECOG ≥2), advanced Ann Arbor stage (III–IV), extranodal involvement ≥2 sites, bone marrow involvement, anemia (hemoglobin <12 g/L), thrombocytopenia (platelet count $< 100 \times 10^9$/L), high erythrocyte sedimentation rate (>40 mm/h), low serum albumin levels, high serum LDH levels, and high β_2m. In addition, bcl-2 protein expression and absence of bcl-6 expression predicted poor OS. IPI also had a high value to predict OS. In the whole series, there was a significant advantage in terms of OS of patients treated with R-CHOP (5-year OS, 48 vs. 71% for CHOP and R-CHOP, respectively; $p < 0.001$) [Figure 1(B)]. The effect of rituximab-containing treatment among different subsets of nodal and extranodal lymphomas is detailed in Table III and depicted in Figure 3. Thus, patients treated with R-CHOP showed longer OS than those receiving CHOP alone only in primary nodal cases (5-year OS: 66 vs. 36%, respectively; $p = 0.0001$).

To further assess the impact of rituximab-containing regimens on OS according to the different primary sites of presentation, multivariate analysis was performed including the most significant variables predicting OS in the univariate analysis, along with the primary site of the lymphoma (nodal vs. extranodal) and the therapy given (CHOP vs. R-CHOP). In the whole group, in the final model with 243 assessable patients, IPI (low- vs. intermediate- vs. high-risk; RR: 0.4 [95% CI: 0.23–0.7]; $p < 0.001$), treatment (CHOP vs. R-CHOP; RR: 1.88 [95% CI: 1.24–2.86]; $p = 0.003$), and primary site of the lymphoma (nodal vs. extranodal; RR: 1.63 [95% CI: 1.05–2.51]; $p = 0.01$) were the most important variables to predict OS. This analysis was performed in primary nodal and extranodal

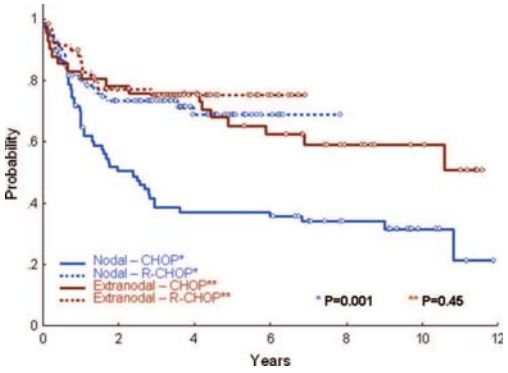

Figure 3. Overall survival of patients with diffuse large B-cell lymphoma according to the primary site of the disease (nodal vs. extranodal) and the therapy given (CHOP vs. rituximab plus CHOP [R-CHOP]).

subgroups. In patients with nodal lymphomas, IPI (RR: 0.58 [95% CI: 0.30–1.15]; $p = 0.01$) and treatment (RR: 2.78 [95% CI: 1.63–4.55]; $p = 0.001$) maintained the prognostic value for OS, whereas in primary extranodal cases only IPI (RR: 0.24 [95% CI: 0.07–0.75]; $p < 0.001$) was important for OS.

In the subset of 78 patients with this information available, neither the expression of single antigens, nor the GCB or non-GCB cell of origin profile, predicted OS before or after R-CHOP. The primary sites did not significantly influence OS in the different subsets.

Discussion

The primary site of the lymphoma, either the lymph node or different extranodal territories, can separate two different groups of DLBCLs, nodal and extranodal, with particular clinico-biological features and different natural history [18]. It has been suggested that genetic differences between nodal and extranodal DLBCLs might exist, including single gene alterations, such as *c-MYC*, *BCL-6*, *REL*, and *FAS* (more frequently seen in extranodal DLBCL) [40]. However, no specific alteration has been described, and, for the moment, the WHO classification does not take into consideration the primary site of the lymphoma for the classification.

Consideration of a lymphoma as primary nodal or extranodal is controversial [12]. Patients with purely nodal or extranodal involvement are easily classified. Although in some studies only localized extranodal lymphomas have been defined as primary extranodal [7,41], this restrictive criterion gives an incomplete picture of these lymphomas. For this reason, those cases with 'clinically relevant' extranodal involvement are usually considered as extranodal [2–4,12,13,18]. Cases with extensive disease, involving both nodal and extranodal areas, are difficult to categorize. In the present report, following previous publications, these cases were included among the nodal lymphomas [12]. This may represent a bias against the nodal group, but does not affect the activity of therapy in the extranodal subgroup.

For decades, the treatment of patients with DLBCL was based on chemotherapy. The addition of rituximab to chemotherapy, so-called immunochemotherapy, dramatically improved the outcome of these patients, as demonstrated both in clinical trials and in retrospective population-based studies [20–24]. Thus, at the present time, immunochemotherapy is considered the gold-standard treatment for any CD20-positive DLBCL [19]. However, since DLBCL is heterogeneous at the molecular level, some authors hypothesize that rituximab improves survival mostly in certain subgroups of DLBCLs. In fact, different reports have pointed out that the positive effect of adding rituximab might be only marginal in specific subsets of patients. For instance, there are data supporting that only patients with bcl-2-positive tumors (but not those bcl-2-negative) [25,27,28] or only patients bcl-6-negative (but not those bcl-6-positive) [29] significantly benefit from immunochemotherapy. Moreover, patients with activated B-cell-like (ABC)-DLBCL seemed to benefit much more from adding rituximab than patients with GCB-DLBCL [31]. Certainly, these observations should be confirmed in prospective larger series, and, in fact, recently published data seem to disprove some of these assertions [32,42].

The present study confirms that the use of immunochemotherapy dramatically improved both the CR rate and OS in an unselected series of DLBCL patients from a single institution. Interestingly, when analyzing the results according to the different primary sites of the disease, we found that the benefit of adding rituximab mainly affected nodal lymphomas, whereas it was much lower in primary extranodal cases. In fact, the use of immunochemotherapy equalized the prognosis of nodal and extranodal DLBCL (Figures 2 and 3). These findings were not related to the initial risk of the patient, as measured by Ann Arbor stage or IPI. Several reasons could be invoked to explain the present findings. The subset analysis tends to fractionate the series into small groups, in which it would be more difficult to reach statistical significance. On the other hand, patients with WR and GI DLBCLs had such a good outcome when treated with chemotherapy alone that it is very difficult to demonstrate an improvement of the results. In this sense, the number of patients with extranodal DLBCL in the current series is not powered to discard a benefit of

rituximab. Moreover, although the initial features of the patients before and after the rituximab era were similar, the historical comparison is a potential weakness of the present study. Anyhow, it would be interesting to speculate whether this relative lack of effectiveness of immunochemotherapy could reflect some biological particularities of extranodal DLBCL. Thus, recent publications [42,43] have pointed out the importance of the microenvironment in tumor invasion, disease progression, and clinical outcome of patients with DLBCL, but no information is available on how the microenvironment could affect the response to immunochemotherapy. There are no data on how the stromal gene signatures could be differentially expressed in nodal and extranodal DLBCLs.

We have stressed in this study that the benefit of rituximab in patients with primary extranodal DLBCL was clearly lower than in patients with nodal DLBCL. We consider that this information is interesting, and worthwhile communicating. However, to change the current gold-standard for all patients with DLBCL, R-chemotherapy, and to perform a new randomized trial in extranodal cases is not realistic. Nevertheless, the retrospective analysis of patients included in the large randomized trials according to the nodal vs. extranodal origin would be of interest. In addition, it would be important to take into consideration the primary origin status (nodal vs. extranodal) of the lymphoma in future trials.

In conclusion, the primary site of the disease was associated with particular clinico-biological features and outcome in patients with DLBCL in the present series. The use of immunochemotherapy dramatically increased the outcome of nodal DLBCL, whereas its effect was much lower in primary extranodal cases, so the prognosis of patients with nodal and extranodal lymphomas has been equalized in the rituximab era.

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Gene-expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy

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Diffuse large B-cell lymphomas (DLBCLs) can be divided into germinal-center B cell–like (GCB) and activated-B cell–like (ABC) subtypes by gene-expression profiling (GEP), with the latter showing a poorer outcome. Although this classification can be mimicked by different immunostaining algorithms, their reliability is the object of controversy. We constructed tissue microarrays with samples of 157 DLBCL patients homogeneously treated with immunochemotherapy to apply the following algorithms: Colomo (MUM1/IRF4, CD10, and BCL6 antigens), Hans (CD10, BCL6, and MUM1/IRF4), Muris (CD10 and MUM1/IRF4 plus BCL2), Choi (GCET1, MUM1/IRF4, CD10, FOXP1, and BCL6), and Tally (CD10, GCET1, MUM1/IRF4, FOXP1, and LMO2). GEP information was available in 62 cases. The proportion of misclassified cases by immunohistochemistry compared with GEP was higher when defining the GCB subset: 41%, 48%, 30%, 60%, and 40% for Colomo, Hans, Muris, Choi, and Tally, respectively. Whereas the GEP groups showed significantly different 5-year progression-free survival (76% vs 31% for GCB and activated DLBCL) and overall survival (80% vs 45%), none of the immunostaining algorithms was able to retain the prognostic impact of the groups (GCB vs non-GCB). In conclusion, stratification based on immunostaining algorithms should be used with caution in guiding therapy, even in clinical trials. (*Blood*. 2011;117(18):4836-4843)

Introduction

Diffuse large B-cell lymphoma (DLBCL), although considered a single category in the World Health Organization classification, most likely includes different clinicopathologic entities difficult to separate using standard techniques.[1,2] From the clinical standpoint, the introduction of immunochemotherapy in the treatment of DLBCL has dramatically improved the outcome of these patients compared with chemotherapy alone.[3-8] However, a significant proportion of these patients (20%-30%) become refractory or eventually relapse.[9] Therefore, the identification of factors, either biologic or clinical, that can identify patients at a higher risk is a priority. Different prognostic factors for response and survival have been described for DLBCL, but in the rituximab era, the role of these biologic prognostic factors has yet to be determined.[10]

DLBCLs can be divided by gene-expression profiling (GEP) studies into germinal center B cell–like (GCB) and activated B cell–like (ABC) subtypes, with the latter having a significantly poorer outcome than the GCB group.[11] These molecular subtypes are associated with different outcomes, even after the introduction of immunochemotherapy.[12] However, GEP techniques are not applicable to the routine clinical practice, and different approaches using immunophenotypic algorithms with small panels of biomarkers have been developed to translate the robust information from molecular studies into a routine clinical platform.[13-17] Two of these algorithms were designed in the preimmunotherapy era and the other 3 were used in cohorts of patients treated with rituximab. The biomarkers used included the GCB markers CD10, BCL6, GCET1, and LMO2 and antigens related to postgerminal center (non-GCB) differentiation, such as MUM1/IRF4 and FOXP1. In addition, the categorization of DLBCL into a GCB or non-GCB subgroup was shown to be associated with clinicopathologic features of tumors and predicted patient outcome in some studies but not in others.[13-23] The reasons for these contradictory results may be complex, and include difficulties in the standardization of both the staining methodologies and evaluation of the results[24] and heterogeneity in the characteristics of the patients in the different studies.[13-18] However, it is not clear whether any of the algorithms is superior to the others in obtaining GEP molecular information, because a comparative study using all of them has not been performed and only 3 studies have associated the immunophenotypic algorithm with the GEP molecular classification.

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Table 1. Initial and evolutive features of 157 patients with DLBCL

Feature	n (%)
Age	
median (range)	65 (17-91)
≤ 60 y	65 (41%)
Sex	
Female	80 (51%)
Male	77 (49%)
Poor performance status (ECOG-PS> 1)	66 (42%)
B-symptoms	55 (35%)
Extranodal involvement	63 (40%)
Bone marrow involvement	46 (29%)
Ann Arbor stage III-IV	94 (60%)
High serum LDH	93 (59%)
High serum β2-m*	64 (47%)
IPI	
Low risk	47 (30%)
Low/intermediate risk	35 (22%)
High/intermediate risk	38 (25%)
High risk	37 (23%)
Response to therapy	
CR	119 (76%)
Partial response	4 (2%)
Nonresponse/progression	34 (22%)

*β2m levels were available in 135 cases.
ECOG-PS indicates Eastern Cooperative Oncology Group performance status.

The aim of the present study was to compare the above-mentioned algorithms in a series of patients with DLBCL homogeneously treated with immunochemotherapy to assess their correlation with GEP data and their usefulness in predicting patient outcome.

Methods

Patients

Two-hundred eighty-seven patients were diagnosed with DLBCL from January 2002 to December 2006 in 5 institutions from the Grup per l'Estudi dels Limfomes de Catalunya i Balears (GELCAB). Patients with a recognized disease phase of follicular lymphoma or another type of indolent lymphoma with subsequent transformation into a DLBCL, as well as those with immunodeficiency-associated tumors, posttransplant lymphoproliferative disorders, and those with intravascular, central nervous system, primary effusion, or primary mediastinum lymphomas were excluded from the study. In 157 of 287 patients, the material necessary to construct a tissue microarray (TMA) and to assess the expression of the different antigens was available. These patients constituted the subjects of the present study. No significant differences were observed regarding main initial features and outcome between the 157 patients with available TMA and the remainder (data not shown). The study was approved by the Ethical Committee of the Hospital Clínic, University of Barcelona. Informed consent was obtained in accordance with the Declaration of Helsinki.

Staging measures included patient history and physical examination; blood cell counts; serum biochemistry, including lactate dehydrogenase (LDH) and β2-microglobulin (β2m) levels; chest, abdomen, and pelvis computerized tomography scans; and bone marrow biopsy. Posttherapy restaging consisted of a repetition of the previous tests and/or biopsies. Response was assessed according to conventional criteria.[25]

The median age of the patients was 65 years (range 17-91) and the male/female distribution was 77/80. The main characteristics of the patients are listed in Table 1. All patients received rituximab-containing chemotherapies, including regimens with anthracyclines in 133 (85%) patients. Response to treatment was as follows: 119 (76%) complete responses (CRs), 4 partial responses (2%), and 34 nonresponses (22%). The median follow-up for surviving patients was 4.3 years (range 0.8-8.6). The 5-year progression free-survival (PFS) of the series was 50% (95% CI: 42%-58%). Sixty-one patients died during the follow-up, with a 5-year overall survival (OS) of 58% (95% CI: 50%-66%). PFS and OS curves are shown in Figure 1. The main initial and evolutive variables, including the histologic parameters indicated in "Histologic review and TMA construction," were recorded and analyzed for prognosis.

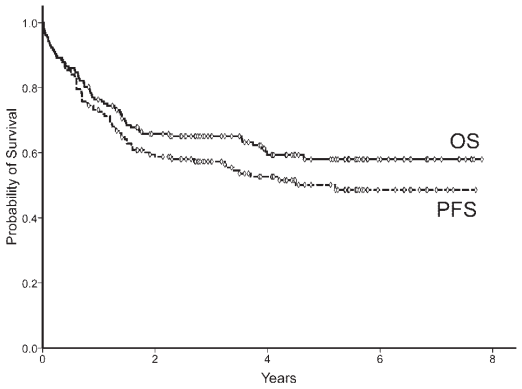

Figure 1. PFS and OS of 157 patients with DLBCL.

Histologic review and TMA construction

The diagnosis of DLBCL was based on the criteria established in the World Health Organization classification.[1] The diagnostic samples were reviewed by expert hematopathologists from the 5 hospitals of the GELCAB involved in the study. All immunohistochemical studies were performed after constructing TMAs, including two 1-mm representative cores of each case using a tissue arrayer (MTA I; Beecher Instruments). Standardized methods for tissue fixation (10% buffered formalin) and processing were used at all participating centers. The immunostains were performed on formalin-fixed, paraffin-embedded tissue sections using a fully automated immunostainer (Bond-Max; Vision BioSystems). All immunostains were centralized in one of the centers involved in the study and evaluated in common in a multiheaded microscope. The conditions and antibodies are shown in Table 2. CD10, BCL6 (kindly provided by Dr M. A. Piris, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain), MUM1/IRF4, BCL2, FOXP1 (kindly provided by Dr A. H. Banham, John Radcliffe Hospital, Oxford, United Kingdom), GCET1 (kindly provided by Dr M. A. Piris), and LMO2 were the markers used to build the algorithms. The assessment of CD10, BCL6, MUM1/IRF4, and BCL2 was performed according to the original studies describing the algorithms and following the recent guidelines recommended for their interpretation by the Lunenburg Lymphoma Biomarker Consortium. Appropriate internal controls were necessary to evaluate the immunostains.[26] FOXP1, GCET1, and LMO2 were evaluated based on previous publications.[27-29] Endothelial cells were used as internal controls for FOXP1 and LMO2. For GCET1, tonsil sections were used as an external control.

The diagnostic samples and immunohistochemical studies were reviewed by at least 3 expert hematopathologists blinded to the clinical details. The cores were evaluated in a semiquantitative manner, and all markers used to build the algorithms were dichotomized at a positive cutoff that depended on the algorithm used. Small biopsy samples that could not be included in a TMA and cores that dropped off of the TMAs were studied as whole-tissue sections. For individual cores with discordant results, the core with the most representative and highest number of positive cells was scored. The discrepancies between the observers were resolved by reaching consensus.

In addition to the markers included in the immunophenotypic algorithms, we also studied the new GCB markers HGAL, IRF8, and JAW1 and the non-GCB markers XBP1 and BLIMP1. These additional biomarkers

Table 2. Immunophenotyping study: antibodies and conditions of use

Antigen	Source	Dilution	Incubation, min
CD20/CD79a	Dako	1:80	30
CD10	Novocastra	1:25	30
BCL6	Centro Nacional de Investigaciones Oncológicas	1:25	60
GCET1	Centro Nacional de Investigaciones Oncológicas	1:1	30
HGAL	Dako	1:500	60
IRF8	Santa Cruz Biotechnology Sc 13043 rabbit polyclonal	1:100	60
LMO2	Santa Cruz Biotechnology Sc 65736 mouse monoclonal	1:4000	60
JAW1	Santa Cruz Biotechnology Sc 11688 goat polyclonal	1:100	60
MUM1/IRF4	Dako	1:400	60
FOXP1	A. H. Banham	1:80	30
BLIMP1	Santa Cruz Biotechnology Sc 13206	1:2	60
XBP1	Santa Cruz Biotechnology Sc 7160	1:400	120
BCL2	Dako	1:75	60

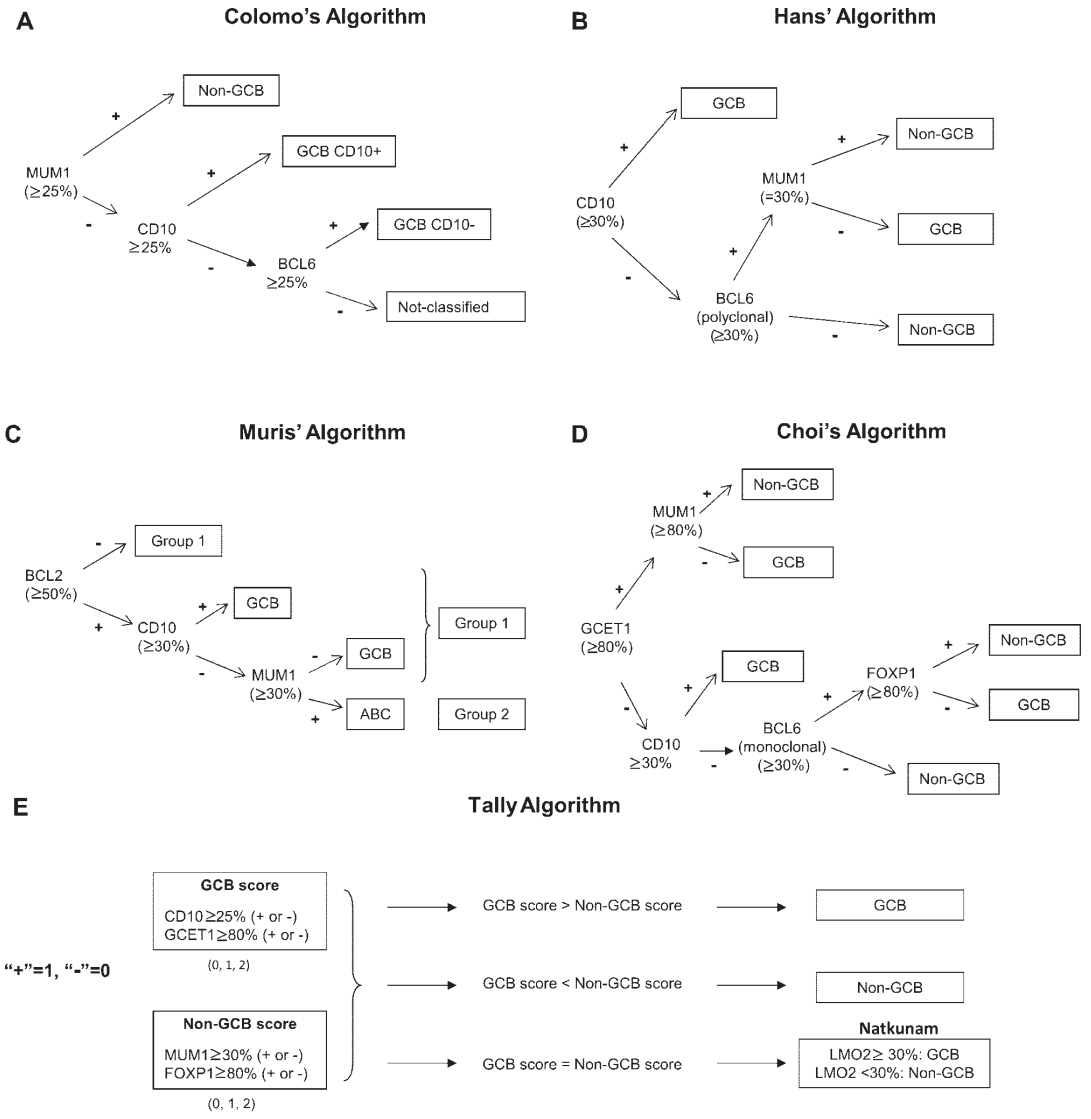

Figure 2. Five immunostaining algorithms used to assess differentiation profile (GCB vs non-GCB) in patients with DLBCL.

Table 3. Immunohistochemical expression of single antigens in 157 patients

Antigen	No. of assessable samples	No. of positive samples (%)
CD10*	157	41 (26%)
BCL6*	146	94 (64%)
GCET1*	146	67 (46%)
HGAL	139	61 (44%)
IRF8	147	42 (29%)
LMO2*	143	60 (42%)
Jaw1	149	126 (85%)
MUM1/IRF4*	148	41 (28%)
FOXP1*	143	112 (78%)
BLIMP1	147	66 (45%)
XBP1	104	28 (27%)
BCL2*	152	76 (50%)

*Antigens used to build up the algorithms.

were evaluated semiquantitatively and the samples were stratified into 5 groups according to the percentages of positive tumor cells: 1 (0%- ≤ 10%), 2 (10%-25%), 3 (26%-50%), 4 (51%-75%), and 5 (≥ 75%). The positive values and cutoff for these new markers was assessed based on previous studies.[29-32]

Immunophenotypic algorithms

To determine the origin of GCB or non-GCB, we applied 5 different algorithms described previously using the following panel of antibodies: CD10, BCL6, MUM1/IRF4, GCET1, FOXP1, LMO2, and BCL2. The thresholds used were those described previously in the literature for each algorithm and are detailed in Figure 2. In the Colomo algorithm (Figure 2A), the non-GCB phenotype was established if MUM1/IRF4 was positive. The cases that were negative for MUM1/IRF4 and positive for CD10 were considered to be the GCB phenotype. Cases negative for MUM1/IRF4 and CD10 and positive for BCL6 were also assigned to the GCB group. Finally, cases negative for the 3 markers were considered as not classified.[13] For the Hans algorithm (Figure 2B), the cases were assigned to the GCB phenotype if CD10 alone or both CD10 and BCL6 were positive. If both CD10 and BCL6 were negative, the case was considered to be of non-GCB origin.[14] In the Muris algorithm (Figure 2C), cases positive for CD10 were assigned to the GCB phenotype. Cases negative for CD10 were differentiated according to MUM1/IRF4 expression into the ABC or the GCB phenotype. Thereafter, BCL2 immunostaining was used to separate 2 different prognostic groups (1 and 2).[15] The 2 steps of the Muris algorithm were used to assess GCB versus ABC status and the prognostic impact of the algorithms was applied. In the Choi algorithm (Figure 2D), the cases positive for MUM1/IRF4 and/or FOXP1 or negative for CD10 and BCL6 were assigned to the non-GCB group. The cases positive for CD10, GCET1 without MUM1 expression, or positive for BCL6 without FOXP1 expression were classified as GCB.[16] In the Tally algorithm (Figure 2E) described recently, the method included an equal number of GCB markers (GCET1 and CD10) and non-GCB markers (FOXP1 and MUM1/IRF4). This algorithm was constructed from the immunophenotype pair with more positive antigens. Because 2 antibodies are used for each type, the LMO2 antigen determines the phenotype (GCB or non-GCB) when the score is equal in the 2 categories.[17]

GEP

A subset of 62 samples with RNA extracted from fresh frozen lymph nodes was investigated using Affymetrix HG U133 plus 2.0 gene expression arrays. All gene expression array data were normalized using MAS5.0 software, and were log2 transformed. We used the Bayesian compound covariate predictor of the ABC/GCB DLBCL described previously.[12,33] All samples predicted as ABC DLBCL at greater than 90% were called ABC DLBCL. The samples that showed less than 10% of probability of being called ABC DLBCL were classified as GCB DLBCL. All the other cases were considered unclassified DLBCL.

Statistical analysis

The definitions of CR, PFS, and OS were according to standard criteria.[25] Categorical data were compared using the Fisher exact test and the 2-sided P value, whereas nonparametric tests were used for ordinal data. The multivariate analysis of the variables predicting response was performed with the logistic regression method.[34] The actuarial survival analysis was performed according to the Kaplan-Meier method, and the curves were compared with the log-rank test.[35] The multivariate analysis for survival was performed using the stepwise proportional hazards model (Cox).[36]

Results

Immunophenotypic profile

The immunohistochemistry expression of single antigens is detailed in Table 3. The distribution of the patients according to the differentiation phenotype (GCB vs non-GCB) after applying the different algorithms is shown in Table 4. The distribution of the cases according to the phenotype was similar for the Colomo and Hans algorithms. In the Choi and Tally algorithms, the proportion of non-GCB cases assigned was significantly higher (non-GCB vs GCB, 67% vs 33%, and 63% vs. 37%, respectively). In the Muris algorithm, the majority of the cases were allocated as the GCB phenotype (GCB vs non-GCB, 57% vs 43%). The Colomo algorithm is the only one of the 5 algorithms that considers a category of "not classified," and 23 tumors were included in this group. Although most of these cases would be categorized in the non-GCB subtype in the other classifiers, we excluded the Colomo algorithm for the comparison between the other algorithms. Therefore, the other 4 algorithms (Hans, Muris, Choi, and Tally) were completely assessed in 135 patients, with 111 cases (82%) being allocated to the same GCB or non-GCB group.

GEP: concordance with immunohistochemistry

GEP data were available for 62 patients: 30 cases were allocated to GCB origin (48%), 22 cases to ABC origin (36%), and 10 cases (16%) were considered unclassified. No differences in terms of initial features and outcome were observed between the patients with GEP information and those without (data not shown).

The relationship between the expression of single antigens and GEP is shown in Table 5. Only CD10 and JAW1 expression were significantly correlated with GCB origin according to GEP ($P = .007$ and $P = .01$, respectively), whereas MUM1/IRF4 expression was correlated with ABC origin ($P = .02$).

Table 4. Distribution, CR rate, PFS, and OS of 157 patients with DLBCL according to immunophenotype (GCB vs non-GCB)

Algorithm	n (%)	CR rate, n (%)	5-year PFS	5-year OS
Colomo				
GCB	53 (44%)	39 (74%)	48%	54%
Non-GCB	68 (56%)	53 (78%)	55%	62%
Hans				
GCB	61 (41%)	47 (77%)	54%	60%
Non-GCB	88 (59%)	67 (76%)	52%	59%
Muris				
GCB	87 (57%)	63 (72%)	48%	57%
Non-GCB	65 (43%)	51 (78%)	56%	63%
Choi				
GCB	45 (33%)	32 (71%)	48%	54%
Non-GCB	90 (67%)	70 (78%)	52%	61%
Tally				
GCB	55 (37%)	45 (82%)	63%	56%
Non-GCB	92 (63%)	65 (71%)	54%	47%

Table 5. Correlation between GEP and different single antigens in 52 DLBCL patients with available microarray data

			GEP	
Antigen		No. of assessable patients	GCB, n (%)	ABC, n (%)
CD10*	(−)	52	19 (47)	21 (53)
	(+)		11 (92)	1 (8)
BCL6	(−)	48	12 (44)	15 (56)
	(+)		15 (71)	6 (36)
MUM1/IRF4†	(−)	49	22 (68)	10 (32)
	(+)		6 (35)	11 (65)
IRF8	(−)	50	21 (64)	12 (34)
	(+)		7 (41)	10 (59)
GCET1	(−)	48	12 (52)	11 (48)
	(+)		16 (64)	9 (36)
GCET2	(−)	50	12 (55)	10 (45)
	(+)		16 (57)	12 (43)
FOXP1	(−)	49	6 (86)	1 (14)
	(+)		21 (50)	21 (50)
LMO2	(−)	48	10 (48)	11 (52)
	(+)		18 (67)	9 (33)
JAW1‡	(−)	50	0 (0)	4 (100)
	(+)		29 (63)	17 (37)
BLIMP1	(−)	49	14 (54)	12 (46)
	(+)		15 (65)	8 (35)
XBP1	(−)	25	10 (63)	6 (37)
	(+)		7 (78)	2 (22)
BCL2	(−)	51	20 (67)	10 (33)
	(+)		9 (43)	12 (21)

*$P = .007$.
†$P = .01$.
‡$P = .02$.

The concordance between the GEP profiles and the patterns of immunohistochemistry as assessed by the 5 algorithms is shown in Table 6. A significant correlation was observed between GEP data and the Colomo ($P = .02$), Hans ($P = .015$), Muris ($P = .04$), and Tally ($P = .02$) algorithms, whereas the correlation did not reach significance with Choi algorithm ($P = .1$). A higher percentage of misclassified cases was observed in the GCB than in the non-GCB subgroup. The proportion of GCB-DLBCL cases that were not correctly allocated by immunohistochemistry was 41%, 48%, 30%, 60%, and 46% for the Colomo, Hans, Muris, Choi, and Tally algorithms, respectively. Conversely, the proportion of ABC patients who were not properly assigned according to the different algorithms was 19%, 15%, 38%, 16%, and 20% for the Colomo, Hans, Muris, Choi, and Tally algorithms, respectively. The sensitivity in the GCB group was 59%, 52%, 70%, 40%, and 53% for the Colomo, Hans, Muris, Choi, and Tally algorithms, respectively; the sensitivity in the non-GCB group was 81%, 85%, 62%, 84%, and 80%, respectively.

Clinical significance of GEP and immunohistochemistry profiles

The CR rate of the series was 76%. CR achievement was observed more frequently in patients with absence of B-symptoms, ambulatory performance status, absence of extranodal involvement or bulky mass, normal serum β2-m, adriamycin-containing chemotherapy, and low-risk International Prognostic Index (IPI) scores. Neither single-antigen expression nor the differentiation profile as assessed by the immunohistochemistry algorithms was able to predict CR (Table 4). GEP profiles also did not predict CR.

Seventy-four of 157 patients eventually progressed, including 36 of the 119 patients who reached a CR. The 5-year PFS was 50% (95% CI: 42%-58%; Figure 1). Variables with unfavorable prognostic value for PFS were: presence of B-symptoms, nonambulatory Eastern Cooperative Oncology Group performance status (ECOG-PS > 1), extranodal involvement at ≥ 1 sites, advanced Ann Arbor stage (III-IV), high serum LDH, high serum β2-m levels, and high-risk IPI score. High BCL2 protein expression also predicted poor PFS (5-year PFS 27% vs 56% for BCL2 $> 75\%$ vs $\leq 75\%$, respectively; $P = .003$). In addition, the differentiation profile (GCB vs ABC) as assessed by GEP was able to predict PFS (5-year PFS, 76% vs 31%, respectively; $P = .005$), as shown in Figure 3A. Conversely, the immunophenotypic profiles as assessed by the 5 different algorithms did not show significant prognostic value for PFS (Table 4).

Sixty-one patients died during follow-up, with a 5-year OS of 58% (95% CI: 50%-66%; Figure 1). Unfavorable variables predicting OS were: age > 60 years, poor performance status (ECOG-PS > 1), advanced Ann Arbor stage (III-IV), high serum LDH levels, high serum β2-m level, and high-risk IPI score. Patients treated with adriamycin-containing chemotherapy also showed a significant advantage in terms of OS. Among the single antigens tested, only BCL2 overexpression ($> 75\%$) was correlated with poor OS. Differentiation profile as assessed by GEP (GCB vs ABC) showed a significant prognostic value for OS in the subset of patients with this GEP information, with a 5-year OS of 80% vs 45%, respectively ($P = .03$; Figure 3B). In contrast, none of the profiles assessed by immunostaining was able to predict OS, as shown in Table 4 and Figure 4. When the 2-step Muris algorithm, which included BCL2 immunostaining, was used, the algorithm was also unable to predict OS (5-year OS 59% vs. 48% for groups 1 and 2, respectively; $P = .4$). The results were the same when the study was performed in 133 patients receiving strictly R-CHOP (cyclophosphamide, hydroxydaunorubicin, oncovin, and

Table 6. Concordance between GEP (GCB vs ABC) and the immunohistochemistry patterns (GCB vs non-GCB) as assessed by 5 algorithms in 52 patients with available GEP information

	GEP	
Algorithm	GCB, n (%)	ABC, n (%)
Colomo*		
GCB	13 (59%)	3 (19%)
Non-GCB	9 (41%)	13 (81%)
Hans†		
GCB	15 (52%)	3 (15%)
Non-GCB	14 (48%)	17 (85%)
Muris‡		
GCB	21 (70%)	8 (38%)
Non-GCB	9 (30%)	13 (62%)
Choi§		
GCB	10 (40%)	3 (16%)
Non-GCB	15 (60%)	16 (84%)
Tally¶		
GCB	15 (54%)	4 (20%)
Non-GCB	13 (46%)	16 (80%)

*$P = .02$.
†$P = .02$.
‡$P = .04$.
§$P = .1$.
¶$P = .02$.

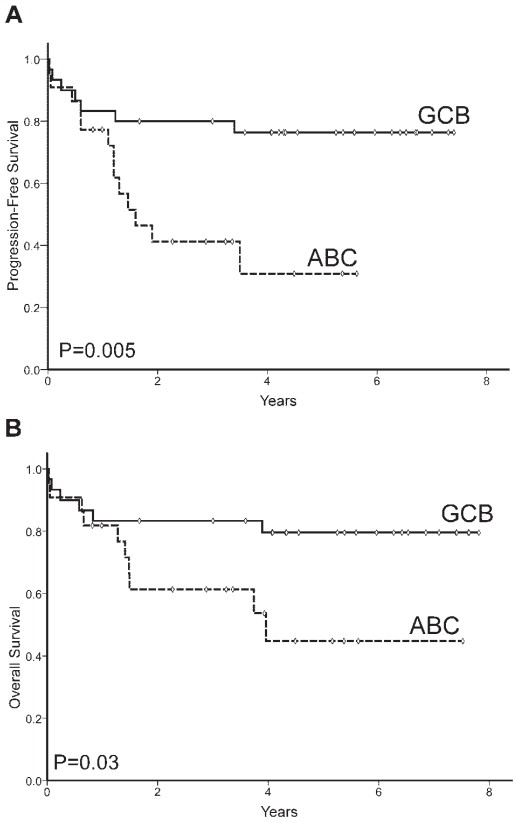

Figure 3. PFS and OS of 52 patients with GEP data available according to molecular subtype (GCB vs ABC) profile.

prednisone/prednisolone plus rituximab; supplemental Figures 1-2, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). A multivariate analysis was performed in the 52 patients with GEP information, including differentiation profile (GCB vs ABC profile), BCL2 expression ($\leq 75\%$ vs $> 75\%$), and IPI score. In the final model with 52 cases, differentiation profile was the most important variable in predicting OS (risk ratio 3.3; $P = .04$).

Discussion

Two types of DLBCL can be recognized on the basis of GEP data: GCB-DLBCL and ABC-DLBCL. These groups have a different cell origin and a distinct clinical behavior, with patients carrying an ABC-DLBCL tumor showing a poorer outcome than patients with a GCB tumor.[11] This fact, originally described in the pre-rituximab era, has been recently confirmed in patients receiving immunochemotherapy.[12] In addition to the prognostic interest, the molecular classification might be useful in selecting treatments that could be active only in specific subtypes of DLBCL, such as the effective use of bortezomib in ABC-DLBCLs.[37-39] Therefore, GEP information is not only of academic interest, but could also be an important decision element in the management of the patients in the near future. Unfortunately, GEP assessment using microarrays is not feasible in routine clinical practice. For this reason, different attempts have been made at capturing the prognostic categorization of GEP information using a more friendly technical approach such as immunohistochemistry. This method can be performed in archival, formalin-fixed, paraffin-embedded tissues and is feasible at any pathology laboratory. Different algorithms have been created combining the expression of well-known antigens, including the ones analyzed in the present study (Colomo, Hans, Muris, Choi, and Tally; Figure 2).[13-17] The real value of these algorithms as a surrogate for GEP data and to define the prognosis of patients with DLBCL is the subject of controversy and was the reason for the present study.

This study was performed in a homogeneous and representative series of patients with DLBCL diagnosed and treated with immunochemotherapy in 5 hospitals from the GELCAB. Clinical features and outcome were as expected for an unselected series of DLBCLs. TMA construction and the assessment of the different antigens were performed based on information from original descriptions.[13-17] Although a significant association between GEP and immunophenotypic profiles was observed (Table 6), the positive and negative predictive values were poor for the 5 algorithms tested. In fact, 30%-50% of GCB-DLBCLs and 15%-25% of ABC-DLBCLs were incorrectly allocated by immunohistochemistry, making its use difficult in clinical practice. Furthermore, in prognostic terms, the applicability of the algorithms was poor in the current series. Although, the number of cases studied by GEP was relative low, the molecular GCB and ABC subtypes showed a clearly differentiated outcome in terms of PFS and OS. As reflected in Figure 4, none of the 5 algorithms was capable of defining groups with prognostic impact.

The current results contradict other studies supporting an excellent correlation between immunohistochemistry and GEP in terms of prognosis.[14,16,17] However, some others studies agree with ours in regard to the lack of clinical significance of the immunophenotypic profiles in patients treated with immunochemotherapy.[18] Several factors may account for these discrepancies, including the population studied and the methodology used. First, retrospective analysis of a heterogeneous series with different therapies may have confounded the results. However, the present study was an unbiased, population-based series, with patients homogenously treated with immunochemotherapy. On the other hand, we have included both nodal and extranodal DLBCLs. The main clinical features, including IPI scores, seem to be very similar to other publications.[17] Secondly, technical shortcomings and interobserver discrepancies in the interpretations of the immunostaining slides most likely have an important role in the divergent data. The liability of immunohistochemistry has been pointed out in several studies, including the Lunenburg Lymphoma Biomarker Consortium study, which was an important step forward in standardizing the immunohistochemical studies of these lymphomas.[26] In our study, we followed the Lunenburg indications to evaluate the antigens described in the consortium. However, some of the antigens used to build up the algorithms are relatively new and the evaluation criteria have not been thoroughly investigated. Therefore, we cannot rule out the possibility that differences in the evaluation of the immunostaining with the newer markers could be part of the discrepancy compared with more recently described algorithms. The low-positive values of markers considered absolutely typical of GCB (ie, GCET1 and LMO2) were positive in 64% and 67% of the cases, respectively, and for the ABC subtype

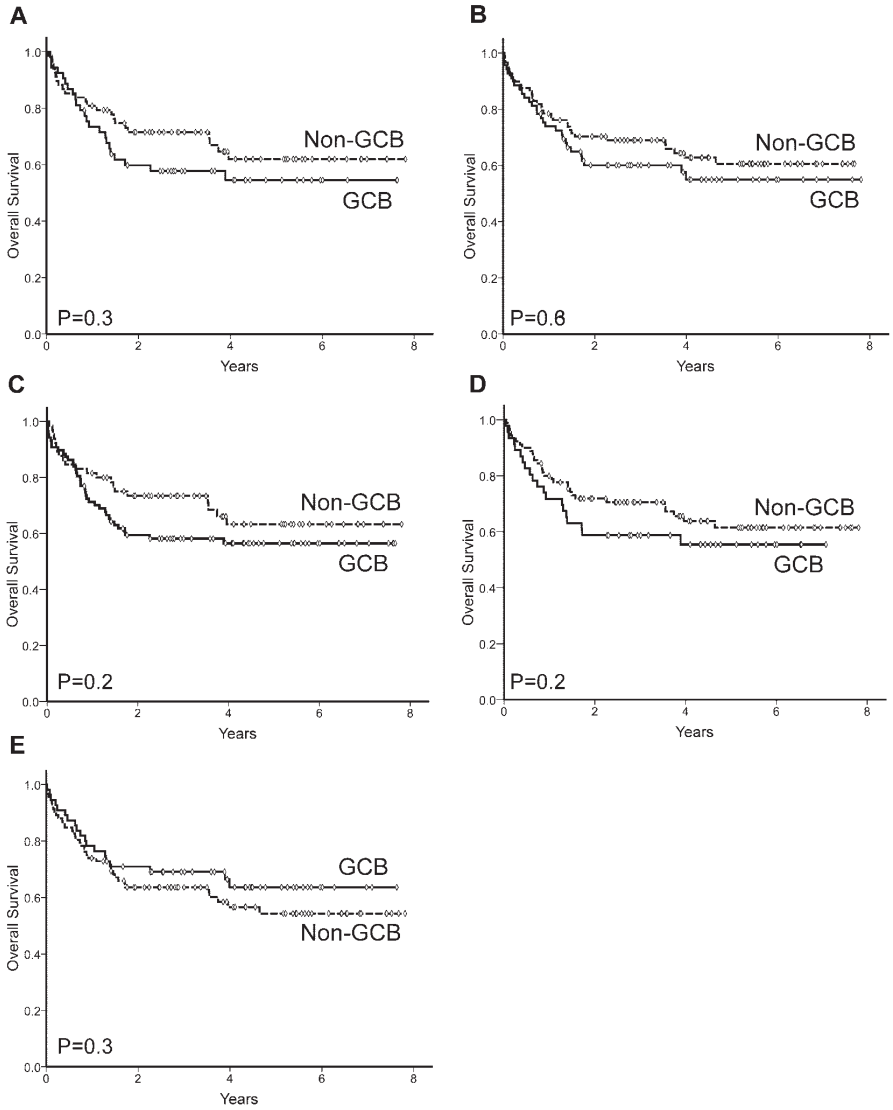

Figure 4. OS of 157 patients with DLBCL according to the differentiation profile (GCB vs non-GCB) as assessed by 5 immunohistochemistry algorithms. (A) Colomo algorithm, (B) Hans algorithm, (C) Muris algorithm, (D) Choi algorithm, and (E) Tally algorithm.

(ie, FOXP1) were positive in 50% of the cases (Table 5). However, we also observed discrepant results using the more established algorithms, suggesting that the reasons for the discordance may not be merely attributable to interpretative differences. We believe that one of the most important difficulties in immunohistochemical attempts to reproduce GEP results is the limitation of capturing information obtained from complex gene-expression signatures on a large number of genes using a very small number of antigens. Therefore, to reliably reproduce GEP information and to transfer it to the clinical practice, new technologies, probably different from standard immunohistochemistry, are warranted.

In summary, in the present study, none of the 5 immunohistochemical algorithms was able to accurately predict the GEP subtype or to separate molecular groups with prognostic value. As a consequence, stratification based on immunohistochemical algorithms for guiding therapy should be viewed very cautiously, even in clinical trials.

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A complete list of GELCAB members appears in the online supplemental Appendix.

Authorship

Contribution: G.G.-G., T.C.-S., E.C., L.C., and A.L.-G. designed and performed research, analyzed data, and wrote the paper; F.C., J.L.M., S.S., S.M., A.V., A.M., P.J., M.P., and A.G.-H. analyzed data; E.G.-B., S.M., J.M.S., L.A., L.E., A.M.-T., and E.G. performed research; and N.V. analyzed data and wrote the paper.

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