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Review

Diagnostic and Prognostic Implications of Genetic Lesions in Non-Hodgkin's Lymphoma

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GENERAL CONCEPTS

EVER SINCE the initial observations made by Boveri, unquestionable evidence has been accumulated that human neoplasms derive from damage to the cell DNA [1]. As in human tumours in general, genetic lesions of non-Hodgkin's lymphomas (NHL) involve proto-oncogenes, tumour suppressor genes, and—to a lesser extent—viral infection of the neoplastic clone [2]. Structural lesions of proto-oncogenes convert them into activated pathological variants, the oncogenes, which constitutively promote cell growth. Conversely, bi-allelic inactivation of tumour suppressor genes, occurring most commonly through deletion of one allele and mutation of the other, deprives these genetic loci of their normal ability to suppress cell growth.

At present, all known NHL genetic lesions, thought to be primary events in NHL pathogenesis, lead to proto-oncogene activation through chromosomal translocation [2]. In the most common instance, these translocations involve the proto-oncogene on one chromosome and an antigen receptor locus (namely, the immunoglobulin heavy or light chain loci in B-cell NHL and the T cell receptor loci in T-cell NHL) on the partner chromosome [2]. The functional consequence of this type of translocation lies in the deregulated expression of the proto-oncogene. More rarely, NHL chromosomal translocations may cause the fusion of the proto-oncogene with another gene, causing the formation of a fusion transcript which is translated into a fusion protein displaying novel biochemical properties compared to the wild-type proteins [2]. With one exception, all the proto-oncogenes involved in NHL code for transcription factors or anti-apoptotic genes.

Among tumour suppressor genes, only the *TP53* gene is currently known to be altered in NHL, although several other chromosomal loci are thought to harbour novel tumour suppressor loci relevant to NHL pathogenesis [2]. Finally, viral infection of NHL cells is virtually restricted to the case of Epstein-Barr virus (EBV), human T-cell lymphotropic virus-I (HTLV-I) and human herpes virus-8 (HHV-8).

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The detailed pathobiology of NHL genetic lesions has been recently reviewed elsewhere and does not represent the specific purpose of this review [2, 3]. Rather, this aims at summarising the clinical usefulness of the routine study of NHL genetic lesions in the clinical management of lymphoma.

CLINICAL AND PATHOLOGICAL HETEROGENEITY OF NHL

The term non-Hodgkin's lymphoma (NHL) is inclusive of a vast spectrum of malignant lymphoproliferations derived from mature (i.e. peripheral) lymphoid cells. As such, NHL are distinguished from lymphoid malignancies of precursor lymphoid cells (i.e. acute lymphoblastic leukaemias) or from tumours of terminally differentiated cells of the lymphoid lineage (i.e. multiple myeloma). The extreme degree of NHLs' clinicopathological heterogeneity is a well established notion of clinical oncology. On the one hand, NHLs may assume a variety of morphological patterns, which are thought to reflect the aggressiveness of the tumour. On the other hand, NHLs showing similar morphology may markedly differ in their clinical behaviour, thus suggesting that prognostic subsets may be identified within a given morphological category.

During the last two to three decades, a number of clinicopathological classifications have alternated in the clinical practice of NHL [4–7]. These early classifications were far from defining the extreme heterogeneity of NHL's clinical behaviour, although the Working Formulation for Clinical Usage and the updated Kiel classification [6, 7] made an attempt at integrating clinical (i.e. prognosis), pathological (i.e. morphology) [6] and biological (i.e. immunophenotype) [7] features. Yet, the precise biological rationale for grouping certain NHL types in a defined category remained elusive.

Recently, the Revised European–American Lymphoma (REAL) classification has included the tumour genotype among the classification criteria [8]. Indeed, a large body of evidence accumulated during the last decade has shown that NHL tend to associate with specific genetic lesions representing the primary pathogenetic event. As a result, NHL subgroups previously thought to be nosologically distinct have been combined in a single entity, whereas NHL categories

initially defined as a single uniform group have been dissected into specific subgroups [8].

The formal inclusion of genetic lesions among the diagnostic tools for NHL classification is likely to substantially influence the clinical management and prognosis of these diseases as well as deepen our understanding of the precise link between molecular alterations and the clinical behaviour of the tumour. Such assertion is supported by the well recognised notion that the presence of peculiar genetic lesions has been the guiding criterium for the therapeutic stratification of acute lymphoblastic leukaemia, which has led to major advances in the cure of the disease [9, 10].

MOLECULAR PATHWAYS IN LYMPHOMAGENESIS

The molecular pathogenesis of NHL may be envisaged as the clustering of genetic lesions into multiple distinct molecular pathways, each of which preferentially associates with a given histological category of lymphoma (Table 1). Many NHL genetic lesions, especially those representing primary events in the genetic history of the tumour (i.e. rearrangements of *BCL-1*, *BCL-2*, *BCL-6*, *c-MYC* and *NPM/ALK*) are virtually restricted to one specific NHL category. Conversely, other genetic lesions, namely lesions considered as secondary hits (i.e. *TP53* mutations, 6q deletions), are generally shared by more than one NHL type. It is notable that most genetic lesions identified until now associate with B-cell NHL, whereas the molecular pathogenesis of the more rare T-cell NHL still represents a major challenge to investigators in the field [2]. The following section will outline the known molecular pathways associated with distinct NHL categories recognised by the REAL classification.

Burkitt's lymphoma

Historically, Burkitt's lymphoma (BL) was the first NHL type to be clarified at the pathogenetic level. The cloning of 8q24 breakpoints, leading to *c-MYC* activation in BL, represented the first example of oncogene involvement in tumour-associated chromosomal translocations [11–15]. The *c-MYC* locus, mapping at 8q24, may be involved in three distinct translocations with one of the immunoglobulin loci, including Ig_H, leading to *t*(8;14)(q24;q32); Ig_κ, leading to *t*(2;8)(p11;q24); and Ig_λ, leading to *t*(8;22)(q24;q11). The functional consequence of all these translocations is identical and results in *c-MYC* deregulated expression [16–19]. Throughout the pathological spectrum of NHL, *c-MYC* activation is virtually restricted to cases of BL and to a fraction of AIDS-related NHL [20, 21]. Translocations of *c-MYC* are associated with all BL, independent of the clinical variant (sporadic, endemic, epidemic or AIDS-related), although the form of the translocation differs among sporadic, epidemic, and endemic BL [22]. In addition to *c-MYC* activation, the molecular pathway associated with BL also includes infection by EBV and *TP53* inactivation. EBV infection occurs in all endemic BLs, whereas it is restricted to a fraction (30–40%) of sporadic and epidemic BL [23]. Although EBV infection in BLs is consistently monoclonal [24], suggesting its presence from the early phases of clonal expansion, EBV fails to express its transforming antigens LMP-1 and EBNA-2 [25]. Finally, *TP53* inactivation is detected in 30% of sporadic and endemic BL and in 60% of epidemic cases, independent of the presence of EBV infection [26, 27]. Overall, it is notable that the molecular pathology of AIDS-related BL mimics that of the sporadic BL variant in terms of a similar mechanism of *c-MYC* translocations and low frequency of EBV infection, whereas it is substantially different from that of endemic BL [21, 28].

Based on the model of *c-MYC* translocations, several other recurrent B-cell NHL breakpoints involving an oncogene and an immunoglobulin (Ig) locus, namely *t*(14;18)(q32;q21), *t*(11;14)(q13;q32) and *t*(3;14)(q27;q32), were cloned in succession.

Follicular lymphoma

The cloning of *t*(14;18)(q32;q21), the chromosomal hallmark of follicular lymphoma (FL), led to the identification of the *BCL-2* proto-oncogene [29–32]. *BCL-2*, the expression of which is consistently activated by the chromosomal translocation [33], codes for a mitochondrial protein acting as a controller of apoptosis [34]. In contrast to most other oncoproteins involved in lymphoid neoplasia, which act as regulators of cell growth and proliferation, the function of *BCL-2* is to prevent apoptosis and to promote cell survival [34]. The distribution of *BCL-2* rearrangements among B-cell NHL is

*Table 1. Frequency of genetic lesions in non-Hodgkin's lymphoma**

Histology	*BCL-1*	*BCL-2*	*BCL-6*	*c-MYC*	*NPM/ALK*	*TP53*	EBV	HHV-8	HTLV-I
B-cell NHL									
Small lymphocytic lymphoma	–	–	–	–	–	Rare	–	–	–
MALT lymphomas	–	–	ND	–	–	Rare	–	–	–
Mantle cell lymphoma	70%	–	–	–	–	–	–	–	–
Follicular lymphoma	–	90%	–	–	–	–	–	–	–
Diffuse large cell lymphoma	–	30%	40%	–	–	–	–	–	–
'Transformed' lymphomas†	–	90%	–	–	–	90%	–	–	–
Burkitt's lymphoma	–	–	–	100%	–	30–60%‡	30–100%§	–	–
Body cavity-based lymphoma	–	–	–	–	–	–	90%	100%	–
T-cell NHL									
Anaplastic large cell lymphoma	–	–	–	–	50%	–	–	–	–
Adult T leukaemia/lymphoma	–	–	–	–	–	40%	–	–	100%

*NHL are classified according to the revised European–American Classification (REA; ref. [8]). Only types for which data are available are listed. †Cases arising from the histological transformation of a previous follicular lymphoma. ‡30% in sporadic and endemic cases; 60% in epidemic cases. §30% in sporadic and epidemic cases; 100% in endemic cases.

selective. They are found in the overwhelming majority of FL independent of the cytological variant (small, mixed, small and large, and large cell FL) as well as in a significant proportion of diffuse large cell lymphoma (DLCL). Although it has been postulated that DLCL carrying *BCL-2* rearrangements represent the transformed phase of a pre-existent FL [35], data from our group suggest that *BCL-2* may also represent the primary genetic lesion of a fraction of *de novo* DLCL [36].

Mantle cell lymphoma

Mantle cell lymphoma (MCL) represents a lymphoma category which has only recently been formally recognised with the advent of the REAL classification [8]. Apart from its morphological and immunophenotypic peculiarities, the recognition of MCL as an individual lymphoproliferative disorder is fully justified by its frequent association with a specific genetic lesion, i.e. rearrangements of the *BCL-1* chromosomal site [37–39]. *BCL-1* rearrangements in MCL result from *t*(11;14)(q13;q32), which juxtaposes the *BCL-1* locus to the Ig_H locus. It is now generally agreed that the proto-oncogene activated by the *BCL-1* translocation lies at a distance from the *BCL-1* breakpoint site, approximately 110 Kb telomeric to the major translocation cluster on chromosome 11. This gene was originally termed *PRAD1* and is now designated cyclin D1 (*CCND1*) [40]. *CCND1* is one of several recently identified cyclins regulating G1 cell cycle progression in various cell types and is consistently overexpressed in MCL carrying *BCL-1* rearrangements. The distribution of *BCL-1* rearrangements and *CCND1* activation throughout the histological spectrum of NHL is highly selective, since this genetic lesion is virtually restricted to MCL, of which 70% of cases carry a rearrangement of *BCL-1*. Early reports of *BCL-1* rearrangements in a fraction of small lymphocytic lymphomas (SLL) have been contradicted by the application of strict morphological and immunophenotypic criteria to the diagnosis of *BCL-1* positive SLL cases, suggesting that they were in fact MCL [8].

B-lineage diffuse large cell lymphoma

The molecular pathways associating with B-cell DLCL, the most common B-cell NHL variant in the Western world, is highly complex, reflecting the extreme degree of histological and clinical heterogeneity of the disease. Several mutually exclusive molecular pathways have been identified, which alternatively associate with either *de novo* DLCL or with DLCL derived from the transformation of a pre-existent FL [35, 36]. Notably, there is no clear preferential association of any of these pathways with a given DLCL morphological variant, i.e. large non-cleaved cell lymphoma, diffuse mixed, small and large cell lymphoma, and immunoblastic large cell lymphoma. The most frequent DLCL molecular pathway, occurring in approximately 40% of the cases, associates with rearrangements of *BCL-6*, most frequently in the absence of other known genetic lesions [41–43]. This molecular pathway is virtually restricted to DLCL arising *de novo* without a pre-existent follicular phase. Rearrangements of *BCL-6* arise as the consequence of the translocation between 3q27, the mapping site of *BCL-6*, and a number of other chromosomal sites, including the Ig loci [41–43]. *BCL-6* codes for a zinc finger protein belonging to a class of transcription factors controlling cell proliferation and differentiation and is constitutively expressed in DLCL carrying 3q27 breakpoints [41–43].

A second genetic pathway detected in B-cell DLCL involves the combination of *BCL-2* rearrangement and *TP53* mutation [44]. This pathway, also known as the 'transformation pathway', associates with the overwhelming majority of B-DLCL transformed from a pre-existent follicular phase. Whereas the *BCL-2* lesion is present from the FL phase, *TP53* mutation is acquired during histological progression to DLCL [44]. Notably, however, *BCL-2* rearrangements may also be detected in the absence of *TP53* alterations in a fraction of *de novo* DLCL, thus suggesting the existence of a distinct pathogenetic sequence [36]. The presence of further genetic heterogeneity is indicated by the fact that 30% of B-cell DLCL do not carry any of the genetic alterations listed above. It is thought that this DLCL group associates with one or more distinct molecular pathways, the precise nature of which is presently under study.

Finally, AIDS-related DLCL display peculiar genetic features. EBV infection, which is consistently absent in DLCL of the immunocompetent host [36], occurs in the majority of AIDS-related DLCL [27]. In addition to EBV infection, AIDS-related DLCL carry mutually exclusive *c-MYC* and *BCL-6* rearrangements in 20% of the cases, respectively [27, 45]. *BCL-2* activation is consistently absent in AIDS-related DLCL, suggesting in all cases their *de novo* origin.

Body cavity-based lymphoma

Recently, a novel molecular pathway has been found associated with B-cell NHL growing in liquid phase in the pleural, pericardial and peritoneal body cavities. These NHL, termed body cavity-based lymphomas (BCBL), consistently associate with HHV-8 infection of the tumour clone in the absence of known alterations of proto-oncogenes or tumour suppressor genes [46–49]. Most cases of BCBL occur in the HIV-infected individual, although rare cases have also been described in the immunocompetent host. Intriguingly, AIDS-related BCBL also consistently harbour EBV infection in addition to HHV-8 [47–49]. It is notable that HHV-8 infection is restricted to cases of BCBL when considering the entire spectrum of B-cell and T-cell malignancies recognised by the REAL classification [47, 48].

Genetic lesions shared by multiple B-NHL categories

Concerning tumour suppressor genes, the involvement of the *TP53* gene at 17p13 in BL and B-cell DLCL, transformed from a pre-existent follicular phase, has already been discussed [44]. Several other chromosomal sites, which are recurrently deleted in NHL, are thought to harbour novel tumour suppressor genes relevant to NHL pathogenesis. In recent years, several studies have focused on deletions of the long arm of chromosome 6, which occur throughout the entire B-cell NHL histological spectrum in approximately 30% of the cases. Molecular studies have shown that these deletions cluster into two discrete regions of minimal deletion, mapping to 6q27 and 6q21–q23, respectively [50]. It is notable that the two types of 6q deletion associate with distinct histological categories of B-cell NHL. Precisely, the 6q27 deletion preferentially associates with low and intermediate grade NHL, whereas alterations of 6q21–q23 are restricted to high grade NHL [51].

T-cell NHL

As already stated, the molecular pathogenesis of T-cell NHL is largely obscure. Apart from the consistent infection

by HTLV-I and the frequent mutation of *TP53* in cases of adult T-cell leukaemia/lymphoma of the Caribbean and the Far East [2], *t*(2;5)(p23;q35) is the only cloned genetic lesion of T-cell NHL in the Western world. *t*(2;5)(p23;q35) occurs in most CD30$^+$ T-cell anaplastic large cell lymphoma (ALCL), which predominantly develop in children and young adults. This translocation causes the fusion of the *NPM* (for nucleophosmin) gene, coding for a nucleolar phosphoprotein and mapping to 5q35, with a novel tyrosine kinase, *ALK* (for anaplastic lymphoma kinase), mapping to 2p23 [52]. As a consequence, a novel fusion protein *NPM/ALK* is formed, which retains the promoter of *NPM*, normally active in T-cell, and the catalytic domain of *ALK*, otherwise not expressed in normal T-cells [52]. The case of *NPM/ALK* is unique among NHL genetic lesions, since it constitutes the only example of oncogene activation by fusion protein formation in these neoplasms.

MOLECULAR MARKERS AS TOOLS FOR NHL DIAGNOSIS

From a clinical standpoint, NHL genetic lesions represent molecular markers of disease serving three distinct purposes: (1) they assist and complement morphological diagnosis; (2) they allow analysis of minimal residual disease; and (3) they are indicators of prognosis in some cases. The prognostic value of NHL genetic lesions will be discussed in the following section.

As outlined previously, genetic lesions may serve as tools for NHL diagnosis because of the preferential association between a given genetic lesion and a distinct NHL category. Examples are the associations between lesions of *BCL-1* and MCL, *BCL-2* and FL, *BCL-6* and DLCL, *c-MYC* and BL, as well as HHV-8 infection and BCBL. The practical usefulness of genetic lesions for the correct classification of NHL may be readily demonstrated by specific examples derived from the literature. Thus, when considering non-follicular small cell NHL, the subgrouping of which is a traditionally difficult task for pathologists, *BCL-1* alterations are considered the most specific clue for a diagnosis of MCL. Since failure-free survival and overall survival of MCL are significantly worse than that of other non-follicular small cell NHL, a correct diagnosis of MCL is mandatory for clinical purposes [53, 54]. Similarly, a reliable morphological diagnosis of ALCL should be complemented by the presence of *t*(2;5)(p23;q25) and CD30 expression [55]. Since *t*(2;5)-positive T-cell ALCL is characterised by a relatively favourable prognosis among CD30$^+$ NHL, a correct diagnosis might influence therapeutic choices and prognostic assessment.

The possibility of monitoring the presence of neoplastic cells with a high sensitivity technique, such as polymerase chain reaction (PCR), applied to studies of minimal residual disease represents a major tool for follow-up evaluation of human tumours. In the NHL field, molecular analysis of minimal residual disease is now feasible for routine purposes in the case of *BCL-1* and *BCL-2* alterations. In these two groups of genetic lesions, the relative consistency of breakpoints at the nucleotide level allows a PCR-based strategy by taking advantage of the J_H consensus sequence on chromosome 14 [56–60]. Several studies have focused on the clinical implications of minimal residual disease in FL carrying *BCL-2* rearrangements. PCR studies have shown that morphologically normal peripheral blood and bone marrow from all patients with advanced FL contain a fraction of lymphoma cells after achieving complete clinical remission with conventional chemotherapy, confirming that traditional chemotherapeutic protocols fail to eradicate *BCL-2* positive cells [61, 62]. The residual *BCL-2* positive cells detectable by PCR distribute according to a patchy pattern of bone marrow infiltration, suggesting that single negative PCR results should be interpreted with caution and making the use of bilateral bone marrow biopsy mandatory when evaluating minimal residual disease [63]. Also, *BCL-2* PCR studies have shown that bone marrow and peripheral blood stem cell harvests of FL patients undergoing autologous transplantation procedures contain malignant cells which, upon infusion into the patient, contribute to subsequent relapse [62].

In all the instances reported above, precise diagnosis and detailed evaluation of minimal residual disease are fundamental for therapeutical decisions. In this respect, genetically distinct groups of NHL may be ideally envisaged as distinct diseases requiring distinct therapeutic options. Learning from the recent past in oncohaematology, it should be kept in mind that molecular classification of acute lymphoblastic leukaemia, leading to therapeutic stratification of the various subtypes of the disease, has been instrumental in improving the chances of cure and eradication of this neoplasm [9, 10].

The goal of the application of molecular genetics to the diagnosis of NHL is the establishment of 100% associations between a given genetic lesion and a given lymphoma entity. Despite the major achievement outlined previously, it should be mentioned that more work needs to be done towards this goal. In several NHL categories (e.g. FL and MCL), the associated genetic lesions (i.e. rearrangements of *BCL-2* in FL and rearrangements of *BCL-1* in MCL) are conventionally detectable in the majority, though not in the totality, of cases. Several practical reasons may explain this discrepancy, including the failure of conventional laboratory assays to detect all the possible types of molecular lesions of a given cancer related gene, as well as the possibility of pathological misdiagnosis among subtly different NHL types. However, at another level it cannot be formally excluded that the genetic features of NHL are more heterogeneous than we presently assume.

PROGNOSTIC RELEVANCE OF GENETIC LESIONS IN THE MANAGEMENT OF NHL

Defining novel and independent prognostic indicators for NHL is a major goal of current clinical research. This is especially true for NHL categories displaying a broad range of clinical behaviour, such as B-cell DLCL. B-cell DLCLs represent a potentially curable disease including a heterogeneous group of neoplasms that are treated homogeneously despite the fact that only 50% of patients experience long term disease-free survival. As discussed above, the clinical heterogeneity of B-cell DLCL is matched by a high degree of heterogeneity in the molecular pathogenesis of the disease. In this respect, the presence of a *BCL-6* rearrangement identifies a sizeable subset of cases associated with a distinct molecular pathway and a distinct clinical behaviour. Recently, a large study correlating B-cell DLCL clinical behaviour and genotypic features has demonstrated that *BCL-6* rearrangements do correlate with a favourable clinical outcome and may thus serve as an independent prognostic indicator in patients with this type of lymphoma [64]. Moreover, *BCL-6* rearrangements in B-cell DLCL specifically associate with extranodal presentation of the lymphoma [64]. In contrast to the favourable

prognosis associated with *BCL-6* positive B-cell DLCL, B-cell DLCL cases carrying a *BCL-2* lesion tend to associate with the worst prognosis, whereas *BCL-6* and *BCL-2* negative B-cell DLCL would represent an intermediate prognostic category [64].

Other examples of prognostic relevance of NHL genetic lesions are the favourable prognosis linked to *NPM/ALK* positive ALCL among anaplastic NHL, and the adverse outcome of *BCL-1* positive NHL among diffuse small-cell, non-follicular NHL [52–54].

CONCLUSION

The application of genetic studies to the clinical practice of NHL has already deepened our ability to classify these neoplasms as well as our understanding of the subtle differences in the natural history of genetically distinct NHL subgroups. It may be presumed that improved molecular diagnosis will soon translate into differential therapeutic protocols according to the tumour genotype, thus leading to therapeutic stratification and, hopefully, improved patients' survival. Also, the molecular evaluation of minimal residual disease in the patient's blood/bone marrow as well as in grafts (bone marrow or peripheral blood stem cells) for autologous transplantation procedures is likely to influence therapeutic decisions and the follow-up. In practical terms, it is notable that the advent of PCR technology has made most of the NHL genetic assays feasible for routine diagnosis in cancer centres or haematology divisions in Western Europe and the U.S.A.

It should be recognised that, despite the major advances of the last few years in the field of NHL molecular genetics, specific molecular markers are still lacking for a number of NHL categories recognised by the REAL classification, including small lymphocytic lymphoma, MALT NHL, splenic villous lymphoma and a fraction of DLCLs. Also, the molecular pathogenesis of most, if not all, T-cell NHL is still unknown. Future efforts should be directed at clarifying these issues, especially since some of these NHL types are relatively common in the haemato-oncological practice.

In addition to the current applications of genetic lesions in NHL management, other uses may be developed in the future. Of particular appeal is the possibility that therapy might be directed at correcting the precise genetic lesion responsible for the development of NHLs. Such therapeutic strategy should, by definition, be largely specific for the lymphomatous cells and hence devoid of the major side-effects presently encountered with standard therapeutic regimens. Initial results from *in vitro* studies indicate the potential feasibility of gene targeting in controlling NHL growth and aggressiveness [65–67]. Yet, as with all human neoplasms, many issues must be resolved before the therapeutic use of gene targeting may be introduced in the clinical practice.

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