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Recurrent mutations in genes involved in nuclear factor- κ B signalling in nodal marginal zone lymphoma—diagnostic and therapeutic implications

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Recurrent mutations in genes involved in nuclear factor- κ B signalling in nodal marginal zone lymphoma—diagnostic and therapeutic implications

Aims: To investigate the spectrum of mutations in 20 genes involved in B-cell receptor and/or Toll-like receptor signalling resulting in activation of nuclear factor- κ B (NF- κ B) in 20 nodal marginal zone lymphomas (NMZLs), 20 follicular lymphomas (FLs), and 11 cases of B-cell lymphoma, unclassifiable (BCL-u).

Methods and results: Nodal marginal zone lymphomas were diagnosed according to strict criteria, including the expression of at least one putative marginal zone marker (MNDA and/or IRTA1). Cases that showed features of NMZL but did not fulfil all criteria were included as BCL-u. All FLs were required to have a *BCL2* rearrangement. Mutations were found in: nine NMZLs, with recurrent mutations in *TNFAIP3* and *CD79B*; 12 FLs, with recurrent

mutations in *TNFRSF14*, *TNFAIP3*, and *CARD11*; and five cases of BCL-u, with recurrent mutations in *TNFRSF14*. *TNFRSF14* mutations were present in FL and BCL-u, but not in any of the NMZLs. In the BCL-u group, *TNFRSF14* mutations clustered with a FL immunophenotype.

Conclusions: These results suggest that *TNFRSF14* mutations point towards a diagnosis of FL, and can be used in the sometimes difficult distinction between NMZL and FL, but to apply this in diagnostics would require confirmation in an independent cohort. In addition, the presence or absence of specific mutations in pathways converging on NF- κ B could be important for decisions regarding targeted treatment.

Keywords: diagnosis, follicular lymphoma, nodal marginal zone lymphoma, non-Hodgkin lymphoma, nuclear factor- κ B, *TNFAIP3*, *TNFRSF14*

Introduction

Nodal marginal zone lymphoma (NMZL) is a rare type of low-grade B-cell non-Hodgkin lymphoma that

is currently defined as a primary nodal B-cell neoplasm resembling lymph node involvement by splenic or extranodal marginal zone lymphoma, but without evidence of splenic or extranodal disease.¹ A diagnosis of NMZL is complicated by the fact that little is known about its pathogenesis, which is reflected by a lack of routinely used positive markers for a diagnosis of NMZL. Therefore, NMZL is often a diagnosis of exclusion, resulting in a heterogeneous disease

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category. In particular, the distinction between NMZL and follicular lymphoma (FL) can be problematic.²

Several novel immunohistochemical markers for NMZL have been reported, including IRTA1 and MNDA.^{3–5} Addition of these markers to the usual diagnostic procedures could potentially result in a more reliable diagnosis of NMZL. Along these lines, we have recently described an immunohistochemical algorithm, including both established and novel markers, to help differentiate between NMZL and FL.⁶

The nuclear factor- κ B (NF- κ B) pathway has an important role in B-cell functioning, with signals from different receptors, including B-cell receptor (BCR), tumour necrosis factor (TNF) receptor (TNFR), interleukin-1 receptor (IL1R), and Toll-like receptor (TLR), converging on NF- κ B. In BCR signalling, binding of BCR by antigen causes phosphorylation of CD79A and CD79B, which initiates a signalling cascade involving multiple proteins, including Bruton's tyrosine kinase (BTK) and protein kinase C β . This results in phosphorylation of CARD11,^{7,8} which then associates with bcl-10, which in turn binds MALT1.^{9–14}

The CARD11–bcl-10–MALT1 complex then interacts with TRAF2 and TRAF6, which induces inhibitor of κ B kinase (IKK) complex activation.⁹ The IKK complex phosphorylates NF- κ B inhibitors, causing them to be degraded by the proteasome, thereby exposing nuclear localization signals on NF- κ B dimers, resulting in their sustained nuclear localization and binding of DNA for transcriptional regulation. IL1R and TLRs signal via receptor-bound MYD88, IRAK, and TRAF6, also resulting in IKK complex activation. Stimulation of TNFR causes IKK complex activation via yet another pathway, involving the proteins RIP1, TAK1, and TAB2. In addition to canonical NF- κ B signalling, a non-canonical pathway exists, in which signalling from lymphotoxin B receptor, B-cell activating factor receptor and CD40 activates NF- κ B.

Mutations in genes involved in NF- κ B signalling have been reported in many subtypes of lymphoma,¹⁵ including extranodal and splenic marginal zone lymphomas.^{15–17} As NMZL resembles its extranodal counterparts, the NF- κ B pathway could also be activated in NMZL. Indeed, enrichment of expression of genes involved in NF- κ B signalling has been shown in NMZL.¹⁸ The gene encoding the inhibitor of NF- κ B signalling *TNFAIP3*, has been suggested to be a tumour suppressor gene in extranodal marginal zone lymphomas,^{19–21} and *TNFAIP3* mutations have also been detected in a small series of NMZLs (in three of nine cases).²²

In the study presented here, we investigated 20 strictly defined NMZLs for the presence of mutations in genes involved in BCR and/or TLR signalling

resulting in activation of NF- κ B. We compared these with 20 FLs and a group of 11 cases of B-cell lymphoma, unclassifiable (BCL-u). The latter group had been diagnosed as possible NMZL in routine diagnostics, but did not fulfil the strict criteria for NMZL used in this study.

Knowledge of the presence of NF- κ B-activating mutations in NMZL is of potential benefit for diagnosis and prognostication. In addition, it carries great promise for targeted treatment. Given the importance of BCR–TLR–NF- κ B signalling in many subtypes of lymphoma, an increasing array of drugs are being developed that target specific components of these pathways. Knowledge of the spectrum of mutations occurring in a specific lymphoma has the potential to enable prediction of which drugs this lymphoma will respond to.

Materials and methods

PATIENT SELECTION

Thirty-one cases of possible NMZL and 20 cases of FL diagnosed between 2003 and 2014 were collected from the archive of the Department of Pathology at the Radboud University Medical Centre (Nijmegen, the Netherlands).

This study was reviewed and approved by the local ethical committee (reference number 2011/270).

Clinically, for a diagnosis of NMZL, patients were required to present with nodal disease without extranodal involvement other than the bone marrow. Also, a clinical presentation with Waldenström macroglobulinaemia was an exclusion criterion. The morphological criteria for NMZL were used as previously reported.⁶

For immunohistochemistry, all lymphomas were stained with an extensive panel of germinal centre markers (CD10, bcl-6, LMO2, and HGAL) and putative marginal zone markers (MNDA and IRTA1). As recently described, we used these markers to classify the tumour, according to an algorithm, into one of three categories: (i) NMZL, for lymphomas expressing fewer than two germinal centre markers and either MNDA or IRTA1; (ii) FL, for lymphomas expressing all four germinal centre markers or expressing two or three germinal centre markers in the absence of MNDA and IRTA1; and (iii) low-grade BCL-u, for lymphomas not fitting into one of the two categories described above.⁶ To be included as NMZL in this study, an algorithm result of 'NMZL' was required.

Finally, the presence of a *BCL2* translocation was excluded in all NMZLs by the use of fluorescence *in-*

situ hybridization (FISH) with a split-probe approach. FISH experiments were performed as described previously, by use of a similar protocol with split probes against *BCL2* (Dako Y5407; Dako, Glostrup, Denmark) or *BCL6* (Dako Y5408; Dako).⁶

From the 31 cases of possible NMZL that were selected from the archive, 20 could be included as NMZL in the study after application of the strict criteria indicated above. The other 11 cases fulfilled the clinical, morphological and cytogenetic criteria for NMZL, but were not classified as 'NMZL' by the immunohistochemical algorithm. These cases were included in the study as BCL-u. The clinical features of patients with NMZL, FL and BCL-u are shown in Table 1.

NEXT-GENERATION SEQUENCING

A custom Ion AmpliSeq panel (Life Technologies, Bleiswijk, the Netherlands) was used, consisting of

410 primer combinations, covering the entire coding sequence or specific regions of interest of 20 genes involved in TLR-BCR-NF- κ B signalling (Table S1), achieving a theoretical coverage of 94.4% of the defined targets (Table S2).

Amplicon libraries were generated in two pools with 20 ng of DNA input per pool, according to the manufacturer's instructions (Life Technologies). Sequencing was performed on an Ion Personal Genome Machine 200 (Life Technologies), with one Ion 318 chip per four DNA samples. Figure S1 shows the percentage of amplicons reaching different depths of coverage.

Sequences were analysed with the SEQNEXT software package (JSI Medical Systems, Kippenheim, Germany). Variants were called if the absolute number of variant reads was ≥ 25 , if the ratio of forward versus reverse read directions did not exceed 30%/70%, and if the variant constituted at least 10% of the

Table 1. Patient characteristics

| | NMZL (<i>n</i> = 20) | BCL-u (<i>n</i> = 11) | FL (<i>n</i> = 20) |
|---|-----------------------|------------------------|-----------------------|
| Male/female (<i>n</i>) | 12:8 | 6:5 | 12:8 |
| Median age (years) | 64 | 56 | 59 |
| Tumour localization, <i>n</i> (%) | | | |
| Nodal | 20 (100) | 11 (100) | 19 (95) |
| Extranodal | 0 (0) | 0 (0) | 1 (5) (parotid gland) |
| Ann Arbor stage, <i>n</i> (%) | | | |
| I | 6 (30) | 2 (18) | 1 (5) |
| II | 2 (10) | 3 (27) | 3 (15) |
| III | 3 (15) | 2 (18) | 3 (15) |
| IV | 6 (30) | 2 (18) | 11 (55) |
| Unknown | 3 (15) | 2 (18) | 2 (10) |
| B-symptoms, <i>n</i> (%) | 1/14 (7) | 1/10 (10) | 2/18 (11) |
| Bone marrow involvement, <i>n</i> (%) | 7/15 (47) | 1/10 (10) | 10/16 (63) |
| Concurrent diffuse large B-cell lymphoma at diagnosis, <i>n</i> (%) | 1 (5) | 1 (9) | 0 (0) |
| Transformation during follow-up, <i>n</i> (%) | 2/16 (13) | 0/10 (0) | 7/19 (37) |
| Grade, <i>n</i> (%) | | | |
| 1–2 | NA | NA | 14 (70) |
| 3A | NA | NA | 5 (25) |
| 3B | NA | NA | 1 (5) |

BCL-u, B-cell lymphoma, unclassifiable; FL, follicular lymphoma; NA, not applicable; NMZL, nodal marginal zone lymphoma.

reads. Intronic mutations, mutations in untranslated regions and silent mutations were filtered out by adaptation of the software settings. Variants were called by the software, but variants that were also present in reactive lymph nodes were considered to be single-nucleotide polymorphisms (SNPs) or artefacts, and were excluded. For missense mutations, *in-silico* pathogenicity predictions were performed with POLYPHEN 2.0 (<http://genetics.bwh.harvard.edu/pph2/>), ALIGN-GVGD, and SIFT, by use of the ALAMUT software package (Version 2.1; Interactive Biosoftware, Rouen, France). Variants that were predicted not to be pathogenic by all prediction programs were considered to be benign, and excluded. Mutations were confirmed by conventional Sanger chain-terminating sequencing in duplicate.

STATISTICAL ANALYSIS

Data were analysed with IBM SPSS STATISTICS version 20 (IBM, Armonk, NY, USA). Pearson's chi-square test was used for comparison of categorical variables. Survival analysis was performed by the use of Kaplan–Meier statistics with log-rank tests. Overall survival was defined as the time from date of diagnosis until the time of last follow-up or death. Event-free survival was defined as the time from date of diagnosis until the time of last follow-up or an adverse event (disease progression, relapse, second tumour, or death from any cause). Two-tailed *P*-values of ≤ 0.05 were considered to be statistically significant.

Results

RECURRENT MUTATIONS IN GENES INVOLVED IN BCR–TLR–NF- κ B SIGNALLING

In NMZL, nine of 20 cases (45%) showed mutations in one or more of the genes tested, and in FL 12 of 20 cases (60%) showed mutations, but the genes involved were different.

In NMZL, mutations were most frequently observed in *TNFAIP3* (six cases, 30%) and *CD79B* (two cases, 10%) (Figure 1A; Table 2). In single cases, mutations were observed in *CARD11*, *MYD88*, and *TNIP2*. In FL, mutations in *TNFRSF14* were most frequent (five cases, 25%), followed by *TNFAIP3* (three cases, 15%), and *CARD11* (three cases, 15%) (Figure 1B; Table 3). Mutations in *CD79B*, *MAP3K3* and *MAP3K7* were observed in single cases.

In the BCL-u group, mutations in *TNFRSF14* were most frequent, being present in four of 11 cases (36%) (Figure 1C; Table 4). Single mutations were

observed in *TNFAIP3*, *TRAF3* and *TRAF7* in this group. In all but one case, mutations in *TNFAIP3* and *TNFRSF14* were mutually exclusive.

Mutations in *TNFAIP3* or *TNFRSF14* did not significantly correlate with disease stage, the presence of B-symptoms, overall survival, or progression-free survival (data not shown).

TNFRSF14 MUTATIONS CLUSTER WITH DIAGNOSIS

With the strict criteria for NMZL used in this study, *TNFRSF14* mutations were not observed in any of the NMZLs. In contrast, *TNFRSF14* was the most frequently mutated gene in both the FL group and the BCL-u group, being present in 25% and 36%, respectively.

CLINICOPATHOLOGICAL DIFFERENCES BETWEEN NMZL AND BCL-U

To investigate additional differences between the NMZL and BCL-u groups, we compared the clinicopathological features of these two groups. NMZLs showed a trend towards more frequent bone marrow involvement, with bone marrow involvement in eight of 15 (53%) patients with NMZL, but in only one of 10 (10%) patients with BCL-u ($P = 0.054$).

Rearrangements involving *BCL6* were observed in four of 11 (36%) cases of BCL-u, but in only one of 19 (5%) NMZLs ($P = 0.028$; Figure 1). No clear differences were observed in disease stage, the presence of B-symptoms, or rate of transformation (Table 1). Overall and event-free survival were also similar between the two groups (Figure 2).

Discussion

NF- κ B transcription factors have important roles in multiple cellular processes, including cell survival, maturation, response to stress, and immune responses. Not surprisingly, NF- κ B signalling has a role in oncogenesis; many solid tumours and haematological malignancies show activated NF- κ B,²³ making it an interesting treatment target. Indeed, therapeutic agents that target BCR–TLR–NF- κ B signalling (including bortezomib, carfilzomib, lenalidomide, ibrutinib, and idelalisib) are increasingly becoming available. To select patients who will benefit from targeted treatment, it will be important to have information on mutations in these pathways. This has been most extensively studied in diffuse large B-cell lymphoma (DLBCL), in which ibrutinib

Table 2. Mutations affecting nuclear factor-κB pathway genes in nodal marginal zone lymphoma

| Gene | Sample no. | Nucleotide change ^a | Exon | Allelic frequency (%) | Pathogenicity description ^b |
|---------------------------------|------------|---|------|-----------------------|---|
| <i>TNFAIP3</i> | 1002 | c.2062G>T p.(Glu688*) | 8 | 10 | Known tumour suppressor gene, truncating (nonsense) or frameshift mutations → pathogenic |
| | 1007 | c.346C>T p.(Gln116*) | 3 | 33 | As above |
| | 1013 | c.301_302delinsATGAGGACA p.(Gly101Metfs*41) | 3 | 23 | As above |
| | | c.338_341del p.(Trp113Serfs*9) | 7 | 15 | As above |
| | 1018 | c.426G>A p.(Trp142*) | 3 | 21 | As above |
| | | c.1035C>G p.(Tyr345*) | 7 | 27 | As above |
| | 1031 | c.296_315del p.(Gly99Alafs*34) | 3 | 14 | As above |
| | 1053 | c.751dup p.(Tyr252Leufs*2) | 5 | 10 | As above |
| c.826_827dup p.(Asn277Leufs*11) | | 6 | 18 | As above | |
| <i>CARD11</i> | 1031 | c.1078A>G p.(Met360Val) | 8 | 33 | Known oncogene, missense mutation in coiled coil domain → probably pathogenic |
| <i>CD79B</i> | 1032 | c.586_588del p.(Tyr196del) | 5 | 31 | Known oncogene, deletion and missense mutation in the ITAM domain, which is needed for signal transduction → probably pathogenic |
| | 1050 | c.586T>G p.(Tyr196Asp) | 5 | 46 | As above |
| <i>MYD88</i> | 1052 | c.794T>C p.(Leu265Pro) | 5 | 11 | Oncogene/tumour suppressor gene not known, missense mutation in hotspot, 3/3 prediction programs predict deleterious mutation → probably pathogenic ALIGN-GVGD: class C65 SIFT: deleterious (score 0.01) POLYPHEN 2.0: probably damaging (score 1.000) |
| <i>TNIP2</i> | 1052 | c.787C>T p.(Arg263Trp) | 4 | 49 | Oncogene/tumour suppressor gene not known, missense mutation in hotspot, 3/3 prediction programs predict deleterious mutation → probably pathogenic ALIGN-GVGD: class C65 SIFT: deleterious (score 0) POLYPHEN 2.0: probably damaging (score 1.000) |

^aNumbering according to: NM_001270508.1/ENST00000612899 (*TNFAIP3*); NM_003820.2/ENST00000355716 (*TNFRSF14*); NM_0032415.5/ENST00000396964 (*CARD11*); NM_000626.2/ENST00000006750 (*CD79B*); NM_002468.4/ENST00000396334 (*MYD88*); NM_024309.3/ENST00000315423 (*TNIP2*); NM_003300.3/ENST00000560371 (*TRAF3*); NM_032271.2/ENST00000326181 (*TRAF7*).

^bALIGN-GVGD: Grantham Variation Grantham Deviation; risk classes are C0, C15, C25, C35, C45, C55, and C65, with higher classes indicating a higher risk of a damaging mutation. SIFT: Sorts Intolerant From Tolerant; scores range from 0 to 1, with a score of ≤0.05 predicted to be damaging. POLYPHEN 2.0: scores range from 0 to 1, with higher scores predicted to be more damaging.

are also characterized by lesions in genes involved in NF-κB signalling.¹⁵ Importantly, for a case to be included as NMZL, it had to fulfil strict criteria, including immunohistochemical expression of at least one putative marginal zone marker (MNDA and/or IRTA1). For comparison, we studied 20 cases of typical FL, all with a *BCL2* rearrangement. Finally, we included 11 unclassifiable cases that had been diagnosed as possible NMZL in routine diagnostics, but

could not fulfil the strict criteria used for NMZL in this study.

Overall, we found fewer mutations in NMZLs than in FLs, and the patterns were different. *TNFAIP3* mutations were the most frequent abnormalities detected in NMZLs. *TNFAIP3* mutations have been reported previously in lymphomas, including marginal zone lymphomas,^{16,21,22,25,26} FL,²⁷ and DLBCL.²⁸ Under normal circumstances, *TNFAIP3*

Table 3. Mutations affecting nuclear factor- κ B pathway genes in follicular lymphoma

| Gene | Sample | Mutation ^a | Exon | Allelic frequency (%) | Pathogenicity description ^b |
|-----------------|--------|-----------------------------------|------|-----------------------|--|
| <i>TNFAIP3</i> | 2024 | c.477T>A p.(Tyr159*) | 3 | 77 | Known tumour suppressor gene, truncating (nonsense) or frameshift mutations → pathogenic |
| | 2003 | c.2090_2091del p.(Arg697Ilefs*54) | 9 | 23 | As above |
| | 2048 | c.190_191delinsGA p.(Ile64Asp) | 2 | 54 | Missense mutation, 3/3 prediction programs predict deleterious effect → probably pathogenic ALIGN-GVGD: class C45 SIFT: deleterious (score 0) POLYPHEN 2.0: probably damaging (score 0.999) |
| | | c.101A>G p.(Asn34Ser) | 2 | 56 | Missense mutation, 0/3 prediction programs predict deleterious mutation → probably not pathogenic ALIGN-GVGD: class C0 SIFT: tolerated (score 0.22) POLYPHEN 2.0: benign (score 0.004) |
| <i>TNFRSF14</i> | 2002 | c.36G>A p.(Trp12*) | 1 | 30 | Known tumour suppressor gene, truncating (nonsense) or frameshift mutations → pathogenic |
| | 2009 | c.423C>G p.(Tyr141*) | 4 | 43 | As above |
| | 2003 | c.357_358del p.(Cys119Trpfs*114) | 4 | 19 | As above |
| | | c.71T>A p.(Val24Glu) | 2 | 18 | Missense mutation, 1/3 prediction programs predict deleterious effect → possibly pathogenic ALIGN-GVGD: class C0 SIFT: tolerated (score 0.13) POLYPHEN 2.0: probably damaging (score 0.999) |
| | | c.103T>C p.(Tyr35His) | 2 | 18 | Missense mutation, 0/3 prediction programs predict deleterious effect → probably not pathogenic ALIGN-GVGD: class C0 SIFT: tolerated (score 0.54) POLYPHEN 2.0: benign (score 0.369) |
| | 2012 | c.552G>C p.(Lys184Asn) | 6 | 47 | Missense mutation, 1/3 prediction programs predict deleterious effect → possibly pathogenic ALIGN-GVGD: class C0 SIFT: tolerated (score 0.34) POLYPHEN 2.0: possibly damaging (score 0.588) |
| | 2013 | c.334T>C p.(Ser112Pro) | 4 | 24 | Missense mutation, 1/3 prediction programs predict deleterious effect → possibly pathogenic ALIGN-GVGD: class C0 SIFT: tolerated (score 0.1) POLYPHEN 2.0: probably damaging (score 0.997) |
| <i>CARD11</i> | 2010 | c.644A>T p.(Lys215Met) | 5 | 39 | Known oncogene, missense mutation in coiled coil domain → probably pathogenic |
| | 2018 | c.746A>C p.(Gln249Pro) | 6 | 43 | As above |
| | 2049 | c.752T>C p.(Leu251Pro) | 6 | 37 | As above |
| <i>CD79B</i> | 2013 | c.617_618del p.(Thr206Ilefs*2) | 6 | 28 | Known oncogene, deletion and missense mutation in the ITAM domain, which is needed for signal transduction → probably pathogenic |

Table 3. (Continued)

| Gene | Sample | Mutation ^a | Exon | Allelic frequency (%) | Pathogenicity description ^b |
|---------------|--------|-------------------------|------|-----------------------|---|
| <i>MAP3K3</i> | 2014 | c.1616T>G p.(Phe539Cys) | 16 | 12 | Oncogene/tumour suppressor gene not known, missense mutation, 2/3 prediction programs scores predict deleterious mutation → possibly pathogenic ALIGN-GVGD: class C0 SIFT: deleterious (score 0) POLYPHEN 2.0: probably damaging (score 1.000) |
| <i>MAP3K7</i> | 2001 | c.681A>T p.(Lys227Asn) | 7 | 57 | Oncogene/tumour suppressor gene not known, missense mutation, 2/3 prediction programs scores predict deleterious mutation → possibly pathogenic ALIGN-GVGD: class C0 SIFT: deleterious (score 0) POLYPHEN 2.0: probably damaging (score 0.962) |

^aNumbering according to: NM_001270508.1/ENST00000612899 (*TNFAIP3*); NM_003820.2/ENST00000355716 (*TNFRSF14*); NM_0032415.5/ENST00000396964 (*CARD11*); NM_000626.2/ENST00000006750 (*CD79B*); NM_203351.1/ENST00000361357 (*MAP3K3*); NM_145331.1/ENST00000369329 (*MAP3K7*).

^bALIGN-GVGD: Grantham Variation Grantham Deviation; risk classes are C0, C15, C25, C35, C45, C55, and C65, with higher classes indicating a higher risk of a damaging mutation. SIFT: Sorts Intolerant From Tolerant; scores range from 0 to 1, with a score of ≤0.05 predicted to be damaging. POLYPHEN 2.0: scores ranging from 0 to 1, with higher scores predicted to be more damaging.

functions as an inhibitor of NF-κB signalling at multiple levels. Loss-of-function mutations cause increased activation of NF-κB, resulting in reduced apoptosis and cell proliferation.^{28,29} In FL, *TNFAIP3* mutations have been reported in association with transformation.²⁷

TNFRSF14 was the most frequently mutated gene in both FL and BCL-u. *TNFRSF14* is a member of the TNF receptor superfamily, and triggering of *TNFRSF14* has been suggested to render B cells more susceptible to FAS-induced apoptosis.^{30,31} In line with this, *TNFRSF14* can act as a tumour suppressor in a mechanism whereby mutations cause loss of function and evasion of T-cell-mediated immune surveillance. *TNFRSF14* mutations have been reported in 18–46% of FLs in previous studies and in 22% of DLBCLs.^{31–33} We found a lower percentage of mutated cases (16%), which can be explained by the fact that, in contrast to earlier studies, we did not investigate copy number changes.

TNFRSF14 mutations were found frequently in FL, but not in any of the NMZLs when strict criteria for the latter diagnosis were used. These results suggest that *TNFRSF14* mutations do not constitute a feature of NMZL, and that the presence of these mutations can be of diagnostic help in ruling out a diagnosis of NMZL. Furthermore, the fact that *TNFRSF14* mutations were frequent in the difficult category of BCL-u is promising, because these lymphomas are difficult to distinguish from FL. All but one case of BCL-u with a

TNFRSF14 mutation showed an FL immunophenotype, suggesting that *TNFRSF14* mutations point towards a diagnosis of FL. This is of potential diagnostic use in lymphomas in which differentiation between NMZL and FL is difficult, but the numbers are small, and these results should be confirmed in an independent cohort before their use in diagnostics.

BCL6 rearrangements were found in only a single case of NMZL, but in four of 11 cases of BCL-u. Although the numbers are small, this suggests that *BCL6* rearrangements are rare in strictly defined NMZL.

Interestingly, the association between the absence of *BCL2* translocations in FL and deletions of the chromosomal region containing *TNFRSF14* (1p36) has been reported in patients who typically present with low-stage inguinal disease with morphologically diffuse FL.³⁴ A direct comparison is difficult, however, because, in the current study, we looked at mutations rather than deletions of *TNFRSF14*, which were also frequently detected in FLs with a *BCL2* translocation.

In NMZL and BCL-u, single mutations were detected in *CARD11*, *MYD88*, *TNIP2*, *TRAF3*, and *TRAF7*. *MYD88* is of particular interest, as the L265P mutation in *MYD88* has been described in the large majority of lymphoplasmacytic lymphomas (LPLs). The single NMZL in our series with a *MYD88* mutation showed no plasmacytic differentiation. Also, the patient did not present with a clinical syndrome of hyperviscosity, fitting Waldenström

Table 4. Mutations affecting nuclear factor- κ B pathway genes in B-cell lymphoma, unclassifiable

| Gene | Sample no. | Nucleotide change ^a | Exon | Allelic frequency (%) | Pathogenicity description ^b |
|-----------------|------------|-----------------------------------|------|-----------------------|---|
| <i>TNFAIP3</i> | 1064 | c.2107_2108del p.(Asp703Cysfs*48) | 9 | 36 | Known tumour suppressor gene, truncating (nonsense) or frameshift mutations → pathogenic |
| <i>TNFRSF14</i> | 1028 | c.283C>T p.(Gln95*) | 3 | 20 | Known tumour suppressor gene, truncating (nonsense) or frameshift mutations → pathogenic |
| | 1023 | c.852A>G p.(*284Trpext*78) | 8 | 65 | As above |
| | 1058 | c.477_487del p.(Asp159Glu fs*71) | 5 | 53 | As above |
| | 1020 | c.288C>G p.(Cys96Trp) | 3 | 29 | Missense mutation, 3/3 prediction programs predict deleterious effect → probably pathogenic ALIGN-GVGD: class C65 SIFT: deleterious (score 0) POLYPHEN 2.0: probably damaging (score 1.000) |
| <i>TRAF3</i> | 1023 | c.598G>T p.(Val200Leu) | 6 | 36 | Tumour suppressor gene, 1/3 prediction programs predict possible deleterious effect → possibly pathogenic ALIGN-GVGD: class C0 SIFT: tolerated (score 0.21) POLYPHEN 2.0: possibly damaging (score 0.808) |
| <i>TRAF7</i> | 1058 | c.352C>A p.(Pro118Thr) | 6 | 51 | Oncogene/tumour suppressor gene not known, 1/3 prediction programs predict possible deleterious effect → possibly pathogenic ALIGN-GVGD: class C0 SIFT: tolerated (score 0.08) POLYPHEN 2.0: possibly damaging (score 0.651) |

^aNumbering according to: NM_001270508.1/ENST00000612899 (*TNFAIP3*); NM_003820.2/ENST00000355716 (*TNFRSF14*); NM_003300.3/ENST00000560371 (*TRAF3*); NM_032271.2/ENST00000326181 (*TRAF7*).

^bALIGN-GVGD: Grantham Variation Grantham Deviation; risk classes are C0, C15, C25, C35, C45, C55, and C65, with higher classes indicating a higher risk of a damaging mutation. SIFT: Sorts Intolerant From Tolerant; scores range from 0 to 1, with a score of ≤ 0.05 predicted to be damaging. POLYPHEN 2.0: scores range from 0 to 1, with higher scores predicted to be more damaging.

macroglobulinaemia. We therefore believe that it is justified to classify this lymphoma as an NMZL with a *MYD88* mutation. However, the distinction between LPL and NMZL with a *MYD88* mutation remains difficult, as was emphasized during the 2014 meeting of the European Association for Haematopathology/Society for Hematopathology meeting, in which rare cases of NMZL with a *MYD88* mutation were recognized.³⁵

In this study, we used a targeted approach, following on from the hypothesis that reported mutations in genes involved in NF- κ B signalling could also have a role in NMZL. This targeted approach allowed good coverage of the genes tested, but limits our results to this gene set only. It is likely that other mutations are also important in NMZL pathogenesis, and, accordingly, a recent study that combined whole exome sequencing, deep sequencing of tumour-related genes, a high-resolution SNP array and RNA

sequencing detected frequent abnormalities in JAK-STAT, NOTCH, NF- κ B and TLR signalling, including frequent mutations in *MLL2*, *PTPRD*, and *NOTCH2*.³⁶ In this study, among other genes, recurrent mutations were also detected in *TNFAIP3*, *TNFRSF14*, *MYD88*, *BCL10*, *REL*, *CARD11*, *TRAF3*, and *BIRC3*. This highlights the fact that multiple pathways are deregulated in NMZL, further complicating the prediction of responses to different targeted treatments.

The number of different signalling pathways that can be deregulated in lymphomas and the heterogeneity of mutations described already pose a challenge for therapy prediction, and this is further complicated by other factors influencing responses to specific targeted therapies, including the epigenome and the tumour microenvironment. For the NF- κ B pathway, for example, activation can be caused by mutations, paracrine signalling, and Epstein-Barr virus infection, and it is possible that patients could

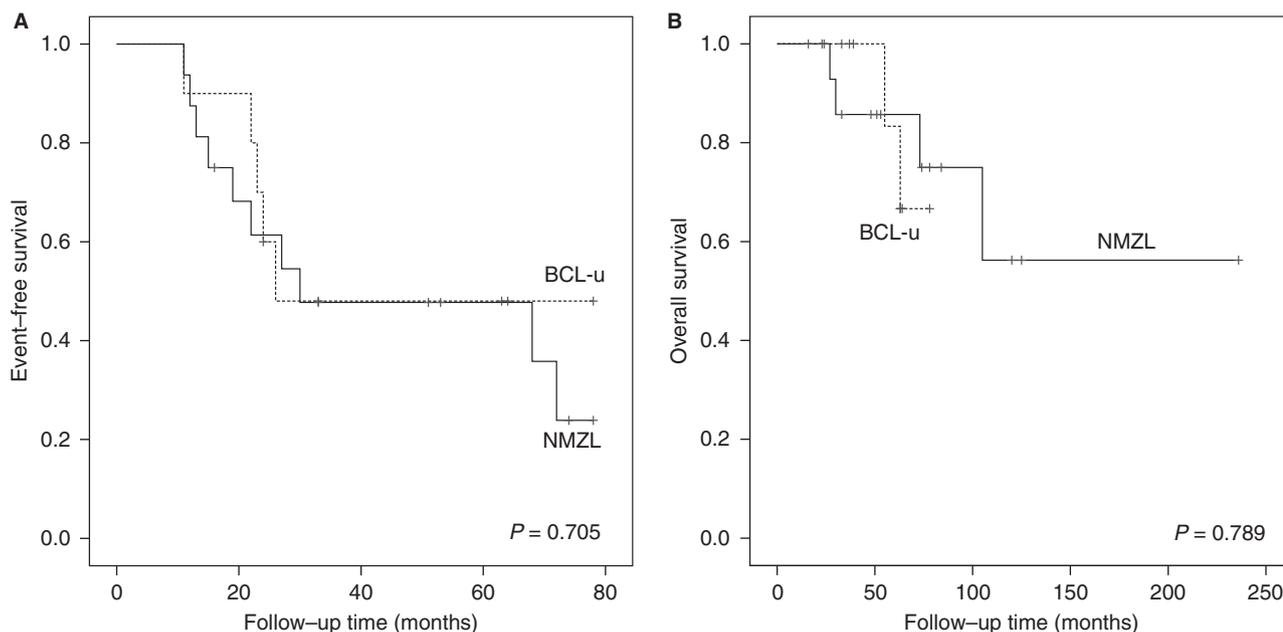


Figure 2. Kaplan–Meier curves for progression-free (A) and overall (B) survival in patients with nodal marginal zone lymphoma (solid line) versus B-cell lymphoma, unclassifiable (dotted line).

benefit from therapy targeting the NF- κ B pathway, regardless of the exact mechanism. Therefore, ultimately, it might be necessary to move towards functional assays rather than mutation detection.

In conclusion, we detected frequent but heterogeneous mutations in genes related to BCR–TLR–NF- κ B signalling in both NMZL and FL. The strong correlation between *TNFRSF14* mutations and an FL immunophenotype is of potential diagnostic use, but confirmation in an independent cohort is warranted. In addition, with the increase in the number of drugs that target pathways that converge on NF- κ B, thorough knowledge of the mutational status of a lymphoma will be helpful to guide targeted treatment decisions.

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Author contributions

M. van den Brand, K. M. Hebeda, B. B. J. Tops, P. J. T. A. Groenen, and J. H. J. M. van Krieken designed the study. M. van den Brand, J. Rijntjes, L. Menting and C. V. Bregitha performed experiments. W. J. F. M. van der Velden and W. B. C. Stevens collected

clinical information. M. van den Brand, P. J. T. A. Groenen and J. H. J. M. van Krieken analysed the results and wrote the paper. All authors reviewed the manuscript.

Conflicts of Interests

The authors declare no potential conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Indication of coverage of amplicons.

Table S1. Primer sequences with genomic coordinates.

Table S2. Theoretical coverage.