The pattern of genomic gains in salivary gland MALT lymphomas

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Funding: the laboratory of M-QD was supported by research grants from the Association for International Cancer Research and Leukaemia Research Fund, United Kingdom. Siebert's laboratory was supported by grants from the Deutsche Krebshilfe.

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Manuscript received April 20, 2006. Manuscript accepted March 22, 2007.

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ABSTRACT

Background and Objectives

Salivary gland mucosa-associated lymphoid tissue (MALT) lymphomas typically lack chromosomal translocations and the molecular genetics underlying their development is unknown. The aim of this study was to investigate chromosomal changes in these lymphomas.

Design and Methods

We performed comparative genomic hybridisation using DNA samples extracted from microdissected tumour cells in 19 salivary gland MALT lymphomas. Recurrent chromosomal changes were further verified by interphase fluorescence *in situ* hybridization (FISH).

Results

Chromosomal gains were much more common than losses. Recurrent gains were found at 1p32-ter (42%), 9q33-34 (84%), 11q11-13 (42%), 17 (58%) and 18q21-22 (42%). Among these, the recurrent gains at 9q34, 11q11-13 and 18q21 were nearly the exclusive gain of the corresponding chromosome. Notably, chromosomal gains at 9q34, 11q13 and 18q21 were frequently concurrent, with 12/19 cases affecting at least two of the three loci. The genomic gains at these chromosomal regions were further confirmed by interphase FISH with probes targeting the *TRAF2* and *CARD9* (9q34), *RELA* and *CCND1* (11q13), and *MALT1* (18q21) loci.

Interpretation and Conclusions

Salivary gland MALT lymphomas show a conserved pattern of chromosomal gains, which appear to target genes positively modulating cell survival and proliferation.

Key words: salivary gland, MALT lymphoma, CGH.

Haematologica 2007; 92:921-927

Primary mucosa-associated lymphoid tissue (MALT) lymphoma of the salivary gland arises on a background of benign lymphoepithelial sialadenitis that is commonly associated with the autoimmune disorder Sjögren's syndrome.[1] The salivary gland MALT lymphoma, as those in other mucosal sites, is usually indolent and responds favorably to treatment. Despite the fact that the majority of salivary gland MALT lymphomas tend to remain localized for a long period of time, even without treatment, a substantial proportion of cases disseminate, particularly to other salivary glands.[2] In addition, salivary gland MALT lymphoma may transform into a diffuse large B-cell lymphoma.[2]

The molecular genetics underlying the development of salivary gland MALT lymphoma is largely unknown. The three chromosomal translocations specifically associated with MALT lymphoma have been examined. t(11;18)(q21;q21) generates a functional API2-MALT1 fusion product and rarely occurs in MALT lymphoma of the salivary gland despite it being frequently seen in those from the lung, stomach and ocular adnexa.[3-5] t(1;14)(p22;q32) brings the entire *BCL10* gene under the transcriptional control of the immunoglobulin gene enhancer and deregulates its expression.[6,7] This translocation is also rarely seen in salivary gland cases.[3,8] t(14;18)(q32;q21) deregulates *MALT1* gene expression [9,10] and its occurrence in primary salivary gland MALT lymphoma remains a subjects of debate. The translocation was found in 5/42 (10%) cases in one study [8] and in 6/28 (21%) in another,[4] but not seen in two more recent investigations that examined 59 and 15 cases.[11,12] t(3;14)(p14;q32) is a newly identified chromosomal translocation in MALT lymphoma, which juxtaposes the *FOXP1* gene to the *IGH* locus and deregulates its expression.[13] The translocation has been frequently found in MALT lymphomas of the thyroid and ocular adnexa, but not in those of the salivary gland.[13]

As MALT lymphoma cells are difficult to grow *in vitro*, only a few cases of salivary gland MALT lymphoma have been studied by conventional cytogenetics.[12,14,15] Nonetheless, studies by both conventional cytogenetic and interphase fluorescence *in situ* hybridization (FISH) indicate that both chromosomal gains, such as trisomies 3 and 18, and chromosomal losses may be frequently present in salivary gland MALT lymphoma.[4,8,14,15] To further investigate such putative chromosomal changes in salivary gland MALT lymphoma, we studied 19 cases using comparative genomic hybridization (CGH), followed by investigations of the recurrent changes using interphase FISH.

Design and Methods

Materials

A total of 19 cases of well-characterized primary salivary gland MALT lymphoma were included in this study. All these cases had been previously studied for t(11;18) by reverse transcriptase polymerase chain reaction (RT-PCR) of the API2-MALT1 fusion transcript and interphase FISH,[3,11] t(1;14) and t(14;18) by Bcl10 and MALT1 immunohistochemistry followed by interphase FISH[11]. All cases were negative for these chromosome translocations. The histology and immunophenotype in each case were reviewed. Paraffin-embedded tumor tissues were available in all cases and fresh frozen tumor tissues were available in two (cases 2 and 17). Use of redundant archival tissues for research was approved by the local ethics committee of the authors' institutions when required (Cambridge University Hospital NHS trust and University College London Hospital NHS trust).

Microdissection and DNA extraction

Tumor cells (500-5000) were microdissected from freshly prepared hematoxylin and eosin (HE)-stained slides of formalin-fixed and paraffin-embedded tissues or fresh frozen lymphoma tissues in each case as described previously.[16] The tumor cells microdissected from paraffin-embedded tissue sections were first treated in 20 μL of 1M sodium thiocyanate at 37°C for 12~16 hours to break the cross-links caused by formalin fixation. After washing three times with phosphate-buffered saline (PBS), the tumor cells were digested with proteinase K (2 mg/mL) in 30 mM Tris-HCl (pH 8.0) buffer containing 10 mM EDTA and 1% sodium dodecyl sulphate at 55°C for 2 days, followed by purification of DNA with the Wizard genomic DNA purification kit (Promega, Southampton, UK). Briefly, the cell digest was mixed with a 1/3 volume of protein precipitation solution and centrifuged. The resulting supernatant was transferred into a fresh tube and genomic DNA was precipitated with isopropanol, washed in 70% ethanol and air-dried. The DNA pellet was dissolved in 20 μL of 10 mM Tris-HCl (pH 8.0). DNA samples were also similarly extracted from whole sections of formalin-fixed paraffin-embedded tonsil tissues and were used as reference DNA in the CGH experiments described below.

DNA amplification and labeling

DNA samples were amplified by degenerate oligonucleotide primer (DOP) PCR using a method described previously.[17] The tumor DNA was labeled with a PCR incorporation reaction using 5 μL of DOP PCR product as template in a total of 50 μL reaction mixture containing 2 U platinum *Taq* DNA polymerase (Invitrogen, Paisley, UK), 1×PCR buffer, 1.5m M $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.1 mM dTTP, 0.02 mM SpectrumRed dUTP (Vysis, Surrey UK), and 1.2 μM DOP primer as described previously.[17] The DNA extracted from whole sections of paraffin-embedded tonsils was similarly labeled with SpectrumGreen dUTP and was used as a reference. Five microliters of the labeling products were routinely analyzed on 1% agarose gels to check the quantity and size of the product. Those with

satisfactory amplification and labeling were purified using QIAquick PCR purification kit (QIAGEN, West Sussex, UK) according to the manufacturer's instructions. Equal amounts (45 μL) of the labeled test and reference DNA were mixed with 25 μL of human Cot I DNA (1 μg/μL, Invitrogen, Paisley, UK), precipitated with ethanol and dried in a vacuum.[17] The DNA pellet was re-dissolved in 10 μL of hybridization buffer (70% formamide, 10% dextran sulfate and 2×SSC, pH 7.0).

CGH and digital image analysis

The normal metaphase spreads were denatured in 70% formamide and 2×SSC (pH 7.0) at 72°C for 2 min and dehydrated in an ethanol series. In the meantime, the mixture of the labeled test and reference DNA was denatured at 77 °C for 5 min, then applied to the metaphase spreads and sealed with a coverslip. The hybridization was carried out at 37°C in a moist chamber for 2 days. After hybridization, the slides were washed in 0.4×SSC/0.3% IGEPAL (Sigma), pH7.0 at 72°C for 2 minutes, then in 2×SSC/0.1% IGEPAL at 42 °C for 5 minutes twice, and finally in distilled water. Slides were mounted using anti-fade medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

Digital images were captured using a cooled CCD-camera (Photometrics, Arizona, USA) with Quips software (Version 3.1.2., Vysis, Richmond Surrey, UK) linked to a Zeiss Axioplan epifluoresence microscope (ZEISS, Jena, Germany). Between six and ten images of high quality metaphases in each case were analyzed using the same software package. The relative genomic gains and losses were determined by comparing the ratio of red (tumor DNA) and green (reference DNA) fluorescence intensity along all chromosomes and the thresholds used for recording chromosomal gains and losses were 1.20 and 0.80, respectively, in accordance with previous studies.[17] The heterochromatic regions, p-arms of acrocentric chromosomes, and the entire X and Y chromosomes were excluded from analysis.

Interphase FISH

For further analysis of the most frequent chromosomal gains (9q34, 11q11-13 and 18q21) found by CGH analysis, we performed interphase FISH as detailed below.

Bioinformatic analysis identified several potential target genes in the commonly gained band 9q34.[18] Among them, *TRAF2* and *CARD9* were selected as targets for the design of FISH probes in view of their interaction with BCL10 and their role in NFκB activation.[19,20] BAC clones RP11-83N9/RP11-100C15 and RP11-251M1 flanking the *CARD9* and *NOTCH1* loci, and BAC clones RP11-83N9/RP11-100C15 together with BAC clone RP11-417A4 which is located telomeric of the *TRAF2* locus, were selected as interphase FISH probes for investigation of chromosomal gain at 9q34.

Similarly, bioinformatic analysis of the genes mapping to 11q11-13 revealed a number of potential target genes. Among them, *RELA* and *CCND1* were selected as targets for FISH probes in view of their respective roles in NFκB activation and cell cycle progression. For *RELA*, interphase FISH was performed using a probe as described previously,[21] while for *CCND1*, interphase FISH was carried out using a commercial LSI Cyclin D1 SpectrumOrange/CEP 11 SpectrumGreen dual color probe (Vysis/Abbott Laboratories Ltd., Berkshire, UK).

For chromosomal gain at 18q21, *MALT1* is likely the gene targeted[6,7,9,10] and interphase FISH was carried out using commercial MALT1 dual color break apart rearrangement probes (Vysis/Abbott Laboratories Ltd, UK).

Bacterial culture, BAC DNA isolation and labeling, and probe preparation were performed as previously described.[18,22] Locus-specific interphase FISH was performed on paraffin-embedded tissue sections essentially as described previously.[3,18] The FISH slides were viewed and the hybridization signals for each probe were counted from 100 cells in each case.

All the above probes were investigated on formalin-fixed and paraffin-embedded tissue sections from eight to ten reactive tonsils to determine the threshold (mean±3 standard deviations) to diagnose chromosomal alterations in lymphoma cells.[23]

Statistical analysis

The statistical difference in the percentage of cells showing three or more copies of a gene locus by interphase FISH among different groups was investigated by the non-parametric Mann-Whitney U test.

Results

Chromosomal gains and losses in salivary gland MALT lymphoma

In general, chromosomal gains were much more common than losses: the former being found in all 19 cases examined, while the latter were only observed in 10/19 cases. Recurrent chromosomal gains were found at 1p32-ter (8/19=42%), 9q33-34 (16/19=84%), 11q11-13 (8/19=42%), 17 (11/19=58%), 18q21-22 (8/19=42%) (Table 1, Figure 1). Among these, the recurrent chromosomal gains at 1p32-ter, 9q33-34, 11q11-13 and 18q21-22 were nearly the exclusive gain of the corresponding chromosome (Table 1, Figure 1). The minimum overlapping region that showed chromosomal gain at 9q33-34 and 18q21-22 appeared to be at 9q34 and 18q21, respectively. Interestingly, the chromosomal gains at 9q34, 11q11-13 and 18q21 were frequently concurrent, with 12/19 cases showing gains at two of the three chromosomal loci.

Confirmation of chromosomal gains at 9q34, 11q11-13 and 18q21 by interphase FISH

To confirm the CGH results, interphase FISH was performed with probes for the *CARD9/NOTCH1/TRAF2* and *CARD9/NOTCH1* loci at 9q34, *RELA* and *CCND1*

Table 1. CGH and interphase FISH findings in salivary gland MALT lymphoma.

Case no.	Chromosomal changes		Percentage cells harboring three or more copies of the gene locus indicated by interphase FISH signals				
	Gains	Losses	9q34 RP11-83N9/RP11-100C15 and RP11-417A4 (CARD9/NOTCH1/TRAF2 BAP) %	9q34 RP11-83N9/RP11-100C15 and RP11-251M1 (CARD9/NOTCH1 BAP) %	11q13 RELA %	11q13 LSI CCND1 %	18q21 LSI MALT1
	The cut-off value (mean±3 standard deviations)		8.4	5.1	4.5	8.57	3.4,
1	2p13		1	4	2	3	2
2*	9q34, 17q		7	11	1	2	2
3	1p32-ter, 6p21, 9q33-ter, 11q11-13, 12q23-ter, 17, 20q	2q22-31	–	–	15	–	0
4#	1p31-ter, 1q32, 5p15, 8q24, 9q33-34, 11p15, 11q11-13, 16p, 17,	4q11-22, 13q21-31	14	21	9	7	1
5	1p31-ter, 4p15-16, 6p21, 9q33-ter, 11q11-13, 17q21-22, 17q24, 20p, 22q	6p23-ter, 9p22-ter, 14q11-12	32	35	–	–	0
6	1p32-ter, 4p15-ter, 9q33-ter, 18q21-ter		–	–	2	1	–
7	4p15-ter, 8q24, 9q33-ter, 11q11-13, 14q24-ter, 17p, 17q12-21, 17q24-ter	11q24	18	11	8	10	1
8#	1p32-33, 3p21, 4p15-16, 6p21, 7q11, 9q32-34, 11q11-13, 12q13, 12q24, 15q22, 16q21-23, 17q, 18q21-22, 19, 20q, 22q	2q21-24, 5q21-23, 6q22	–	–	12	8	73
9	9q34, 10q26, 17q24-25		18	12	2	3	52
10	9q33-34, 10q26, 18q21-22		32	26	7	15	31
11	1p36, 9q33-34, 18q21-22		22	19	4	8	55
12	18q11-21		1	4	1	1	50
13*	9q34, 11p15, 18q21-22		6	7	1	1	38
14	8q21-22, 9q34, 17	9p	11	8	4	3	1
15	1p31-ter, 11q11-13, 15q15-22, 18q22, 22q	6p22, 13q22-31	4	3	8	16	1
16	5q31, 9q34, 11q11-13, 12q24, 17q	2q22-24, 4q21-23, 9p21, 11q23-25, 13q22-32	50	11	–	17	1
17	9q34, 11q11-13, 16q23, 20p		–	-	–	–	–
18	1p32-ter, 8q21, 9q33-ter, 17, 18q21, 22q	8q22-24	2	3	–	–	8
19	1p32-ter, 4p15-ter, 9q32-ter, 11p15, 17, 20q, 22q	6q16-23	–	–	3	3	–

These cases show borderline FISH results with probes targeting 9q34; #these cases show borderline FISH results with probes targeting 11q13; -, not done.

loci at 11q13, and *MALT1* locus at 18q21 on 4 μm paraffin-embedded tissue sections. The cut-off value (mean±3 standard deviations) for *CARD9/NOTCH1/TRAF2*, *CARD9/NOTCH1*, *RELA*, *CCND1* and *MALT1* gene probes are 8.4%, 5.1%, 4.5%, 8.6% and 3.4%, respectively (Table 1). It was noted that in control sections of reactive tonsils, up to 38% (standard deviation = 6.56) of cells showed only one signal with the above FISH probes, indicating truncated nuclei resulting from tissue sectioning. This may have led to a significant underestimation of the true percentages of cells harboring three or four copies of the loci investigated. Interphase FISH with

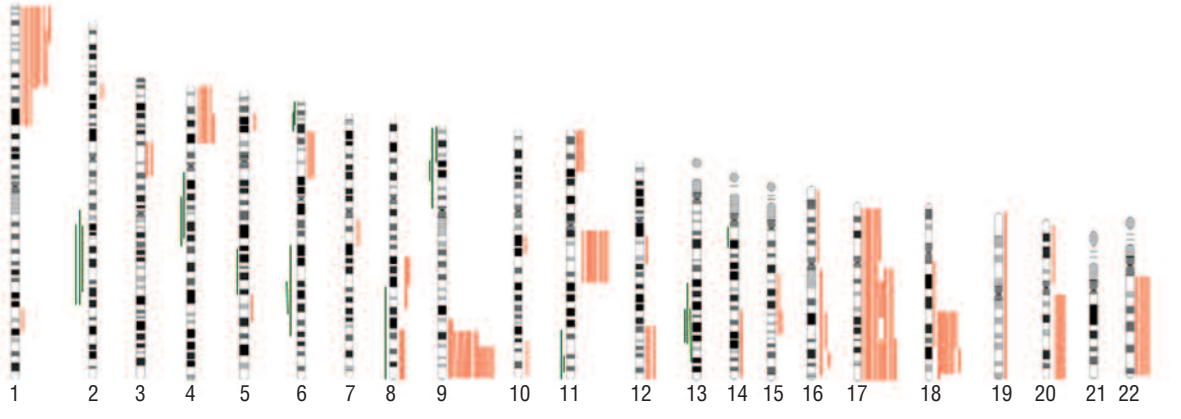

Figure 1. Chromosomal gains and losses in salivary MALT lymphomas. Red lines indicate gains, while green lines indicate losses.

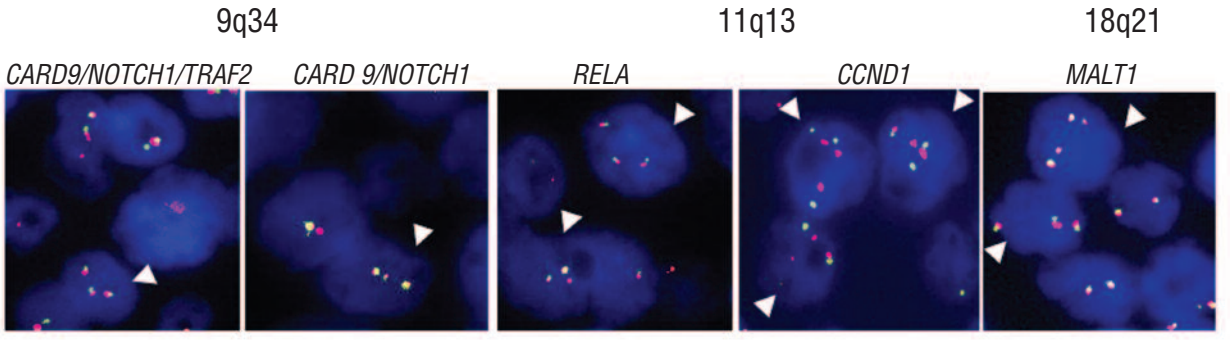

Figure 2. Confirmation of chromosomal gain at 9q34, 11q13 and 18q21 by interphase FISH with probes flanking the *CARD9/NOTCH1/TRAF2* and *CARD9/NOTCH1*, *RELA* and *CCND1*, and *MALT1* loci, respectively. A case (no. 10) with CGH gain at 9q34 showing three co-localized green (BAC clones RP11-83N9/RP11-100C15) and red (RP11-417A4) signals (arrowhead), indicating three copies of the 9q34 region including *CARD9*, *NOTCH1* and *TRAF2*. The same case displays three co-localized green (BAC clones RP11-83N9/RP11-100C15) and red (RP11-251M1) signals (arrowhead) for the probe flanking the *CARD9* and *NOTCH1* genes. A case (no. 3) with CGH gain at 11q11-13 shows three co-localized red and green signals in cells (arrowhead), indicating gain of an extracopy of the *RELA* gene. A case (no. 15) with CGH gain a 11q11-13 shows three red (CCND1) and two or three green signals (centromere probe) in cells (arrowhead), indicating gain of an extracopy of the *CCND1* gene A case (no. 8) with CGH gain at 18q21 shows three co-localized green and red signals with MALT1 probe in cells (arrowhead), indicating gain of an extracopy of the *MALT* gene.

two dual color break apart probes targeting *CARD9/NOTCH1/TRAF2* and *CARD9/NOTCH1* at 9q34 was performed on 14 cases including 11 with CGH evidence of gain at 9q34 and three cases without CGH evidence of gain at this locus. Of the three cases without CGH evidence of chromosomal gain at 9q34, none showed FISH signals indicating three copies of the *CARD9/NOTCH1/TRAF2* or *CARD9/NOTCH1* locus in a proportion of cells well above the threshold (Table 1). In contrast, 8/11 cases with chromosomal gain at 9q34 displayed FISH signals indicating three copies of both the *CARD9/NOTCH1/TRAF2* and *CARD9/NOTCH1* loci in a proportion of cells well above the threshold, with a further two cases (cases 2 and 13) showing a borderline result (Table 1, Figure 2). In support of this, the percentage of cells with three copies of the *CARD9/NOTCH1/ TRAF2* and *CARD9/NOTCH1* loci was significantly (p=0.01; p=0.03 respectively) higher in cases with CGH gain at 9q34 than those without CGH gain at 9q34.

RELA and *CCND1* interphase FISH was performed on 16 cases including six with CGH gain at 11q13 and ten cases without CGH gain at this locus. Of the ten cases without CGH evidence of chromosomal gain at 11q13, only one case (case 10) showed FISH signals of three copies of the *RELA* and *CCND1* loci in a proportion of cells well above the threshold (Table 1). In contrast, four of six cases with gain at 11q13 displayed FISH signals of three copies of the *RELA* and *CCND1* gene in a proportion of cells well above the threshold, with a further two cases (cases 4 and 8) displaying a borderline result (Table 1). The percentage of cells with three copies of the *RELA* locus was significantly (p=0.01) higher in cases with CGH gain at 11q13 than those without CGH gain at this locus. While the percentage of cells with three copies of the *CCND1* gene was much higher in cases with CGH gain at 11q13 than in those without CGH gain at this locus, the difference did not reach a statistical significance. Cyclin D1/CCND1 immunohistochemistry was performed in 16 cases including six cases with gain at 11q13 and none of the cases examined showed a positive staining. *MALT1* interphase FISH was carried out in 16 cases including six with CGH gain at 18q21 and ten cases without CGH gain at this locus. All six cases with gain at 18q21 showed an extra copy of the *MALT1* gene, while one of ten cases without gain at 18q21 displayed an extra copy of the gene in a proportion of cells above the threshold from control. The percentage of cells with three or four copies of the *MALT1* gene was significantly higher in cases with CGH gain at 18q21 than those without (p= 0.004). *MALT1* immunohistochemistry was performed in all 19 cases and no apparent difference in the staining intensity was found between cases with and without gain at 18q21.

In all cases examined by interphase FISH, there was no evidence suggesting gene amplification or chromosomal break at these loci.

Discussion

Several chromosome translocations are associated with MALT lymphomas, but occur at dramatically different frequencies at various mucosal sites. Despite the fact that these translocations involve different oncogenes, they appear to exert their oncogenic activities through a common pathway, thus explaining the homogeneous phenotype of this entity of lymphoma. Mounting evidence indicates that t(11;18), t(1;14) and t(14;18) are molecularly linked by the physiological role of BCL10 and MALT1 in antigen receptor mediated activation of the NFκB pathway.[24] The oncogenic products of these chromosomal translocations have been shown to constitutively activate NFκB, a transcriptional factor for a number of growth factors, cytokines and apoptosis inhibitors.

Salivary gland MALT lymphoma, in common with the majority of MALT lymphomas including those from the

lung and stomach, is typically negative for the above chromosomal translocations. The molecular genetics underlying the development of salivary gland MALT lymphoma is unclear. By screening chromosomal gains and losses using CGH, we showed that salivary gland MALT lymphomas are characterized by recurrent chromosomal gains at 9q34, 11q11-13 and 18q21. Although there was no evidence of gene amplification at these regions, the chromosomal gains were often concurrent.

For chromosomal gain at 18q21, the *MALT1* gene has been proposed to be the target gene or one of the target genes in view of the findings that the MALT1 gene is targeted by chromosomal translocations in MALT lymphoma and is also amplified in cell lines from marginal zone B-cell lymphoma (SSK-41 and Karpas 1718).[10] However, *MALT1* gene amplification is rarely seen in primary lymphoma;[10] rather, a gain of an extra copy of the gene is a common event in t(11;18)(q21;q21)-negative MALT lymphoma, including those of the salivary gland as demonstrated in this and several previous studies.[4,8]

For chromosomal gain at 9q34, *TRAF2* and *CARD9* were likely the genes targeted in view of their interaction with BCL10 and their role in NFκB activation.[19,20] TRAF2 is involved in the activation of the NFκB pathway triggered by both innate and acquired surface immune receptor signalling. During the innate immune responses, TRAF2 transduces signal from the TNF receptor to the NFκB activation pathway.[25] In the adaptive immune responses, TRAF2 may, like TRAF6, activate the IKK complex by polyubiquitination of NEMO (IKKγ) in response to upstream signals from BCL10/MALT1.[25,26] TRAF2 protein is abundantly expressed in various lymphoma cell lines and in Hodgkin/Reed-Sternberg cells of Hodgkin lymphoma, which are characterised by constitutive NKκB acivities.[27,28] CARD9 is a key adaptor in Destin-1 (a major mammalian pattern recognition receptor) mediated NFκB activation and also involved in the activation of the kinases p38 and Jnk in non-TLR-mediated innate immune responses.[29,30] In line with its role in inflammation and immune response, CARD9 is abundantly expressed in gastric MALT lymphoma.[31]

For chromosomal gain at 11q11-13, there are a number of potential target genes, of which *RELA* and *CCND1* (cyclin D1) are likely relevant in the context of the current understanding of MALT lymphomagenesis. RELA is the p65 subunit of the NFκB family and typically forms a heterodimer with p50 or p52, which is the predominant NFκB species in many cell types.[32] Like c-Rel, RELA is also targeted by gene amplification or chromosome translocation in hematologic malignancies, albeit at a much lower frequency.[33] CCND1 is a crucial cyclin for cells to commit DNA synthesis and division, and is frequently targeted by chromosome translocation as well as gene amplification in lymphoid malignancies. There is compelling evidence suggesting a biological co-operation between NFκB and cell cycle regulation in normal and malignant cells. NFκB activation promotes cell cycle progression and suppresses terminal differentiation and these effects occur, at least partially, via NFκB-mediated CCND1 transcription activation.[34]

In summary, salivary gland MALT lymphomas show concurrent chromosomal gains at 9q34, 11q11-13 and 18q21, similar to those seen in t(11;18)(q21;q21) negative gastric MALT lymphoma.[18] The gains at these chromosomal loci appear to target several genes, whose products positively modulate the NFκB pathway (TRAF2, CARD9, RELA, MALT1) or cell cycle (CCND1). The concurrent gain of extra-copies of these positive modulators could play a role in the pathogenesis of translocation negative MALT lymphomas.

Authors' Contributions

YZ: collection of CGH data; HYE: collection of interphase FISH data; JIMS: design and testing FISH probes; SG: design and testing FISH probes; RH: bioinformatic analysis; YJL, RW, JS: CGH; RS: design of FISH probes and study design; PGI: pathology and case contribution; AD: pathology and case contribution; MQD: design of study, data analysis and manuscript writing.

Conflict of Interest

The authors reported no potential conflicts of interest.

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