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MOLECULAR CYTOGENETICS OF LYMPHOMA. WHERE DO WE STAND IN 2010?

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Abstract

For the past 20 years most malignant lymphomas have been classified as clinico-pathologic entities, each with its own combination of clinical, morphologic, immunophenotypic and molecular genetic characteristics. Molecular and cytogenetic abnormalities can be detected by a wide range of techniques, ranging from conventional karyotyping to single nucleotide polymorphism analysis. In this review, we consider the common genetic abnormalities found in lymphoma and discuss the advantages and disadvantage of individual techniques used in their detection. Finally, the authors briefly discuss possible novel developments in the field of lymphoma diagnostics.

For Peer Review

Introduction

During the past two decades molecular diagnostics have become an integral part of the laboratory repertoire in haematopathology. Conventional and molecular cytogenetic techniques are used to detect recurrent chromosomal translocations or breakpoints, as well as specific deletions, gains and point mutations. These abnormalities may be used in the primary classification of a tumour or as prognostic factors. This review aims to give an overview of the currently available molecular and cytogenetic methods, the lymphoma specific targets that can be analyzed by these methods and to provide guidelines for their use in everyday practice. The important molecular abnormalities found in the common types mature B and T cell lymphomas as known in 2010¹ are shown in table 2

Molecular cytogenetic methods.

1. Karyotype analysis / classical cytogenetics.

Only three years after the discovery of the Philadelphia chromosome in 1960,² the first recurrent cytogenetic abnormalities were described in Burkitt lymphoma.³ This was later found to be the classical translocation t(8;14)(q24;q32) in which the *MYC* gene and immunoglobulin heavy chain gene (*IGH*) complex are involved. This was followed by the discovery of many more recurrent chromosomal translocations. Some of these chromosomal translocations are considered as the diagnostic hallmark of specific disease entities, whereas others may be secondary and involved in tumour progression.⁴ Recurrent translocations can be detected in > 90% of the cases of Burkitt lymphoma, mantle cell lymphoma and follicular lymphoma grade 1 and 2, and are considered as the cancer initiating events in these diseases. In other lymphomas, and in particular in diffuse large B cell lymphoma, multiple breakpoints and translocations with a plethora of numerical abnormalities (gains and losses) are seen. In these cases it is often impossible to decide which events are involved in the primary initiation and subsequent progression of the tumour.

A good example of this complexity is the involvement of *MYC* and the 8q24 breakpoint in lymphomagenesis. *MYC* rearrangements are widely regarded as the primary event in Burkitt lymphoma (the t(8;14) or variant t(2;8)(p12;q24) and t(8;22)(q24;q11) translocations), but *MYC* is also frequently involved in tumour progression, either by chromosomal translocation, gain or amplification in other types of lymphoma.. Rearrangements of *MYC* can be found in

5-20% of diffuse large B cell lymphoma. In more than half of these cases additional abnormalities, most commonly the $t(14;18)$, can be identified. These “double-hit” or “dual-hit” lymphomas⁵⁻¹⁰ often have complex cytogenetic alterations including occasional breakpoints at $3q27/BCL6$ (“triple hit” lymphomas). This complexity can be best detected by conventional cytogenetics, since it represents a genome wide method by which many structural and numerical abnormalities can be simultaneously detected, albeit at a low resolution.

The genome wide scope of conventional cytogenetic analysis means that it is often considered to be the “gold standard” for lymphoma diagnostics raising the question as to why is it not part of routine practice. Several logistic and technical problems hamper the routine use of this method. These include the need to obtain fresh tumour tissue which must be processed within few hours after removal from the patient, the need for a relatively large volume of tissue, and the high level of skill required to culture the lymphoma cells, to prepare high quality metaphase cells, and to analyse the often complex karyograms. This makes the method labour intensive, time-consuming and expensive. Moreover, the failure rate is relatively high. In particular in indolent lymphomas and myelomas the tumour cells may fail to grow in culture only normal karyotypes may be seen.

Given these problems the possibility that fluorescent in situ hybridization (FISH) or other methods could completely replace conventional cytogenetics needs to be considered. FISH is a rapid, robust, cheap and relatively easily applicable technique that can be applied to formalin fixed and paraffin embedded (FFPE) tissues and does not need vital, growing cells or specific culture systems (Table 1).^{11, 12} A further advantage is a short turnaround time which is usually in the order of 2 or 3 working days. The major limitation of FISH is that is targeted to the detection of specific abnormalities and the genome wide perspective offered by conventional cytogenetics is lost. This may be clinically important as additional aberrations (or their absence) can be diagnostic or predictive for the course of disease (see Table 2). For example, the diagnosis of Burkitt lymphoma depends not only on the presence of a *MYC* or $8q24$ breakpoint in Burkitt lymphoma, but on the absence of complex genomic complexity abnormalities. 30-40% of all Burkitt lymphoma cases harbour only the $t(8;14)$ or its variants, the rest exhibiting only one to two additional abnormalities (mostly alterations at $1q$, trisomy7, trisomy12 or alterations at $13q$ involving the microRNA 17-92 cluster) are found.^{4, 13, 14} In contrast, the genomic complexity in DLBCL with an $8q24$ breakpoint is usually much

higher.^{13, 15-17} In a recent study of aggressive B cell lymphomas with a *MYC-IG* breakpoint, 22 Burkitt lymphomas had a mean of 1.7 additional conventional cytogenetic abnormalities, whereas 12 other (non Burkitt) lymphomas with a *MYC-IG* breakpoint had a mean of 8.8 abnormalities with three DLBCL showing over 21 cytogenetic abnormalities.¹⁶ In another study using FISH and array CGH on a large series of lymphomas, an almost similar genomic complexity of 2 additional abnormalities in the 39 molecular Burkitt lymphomas versus 9 in the 35 other aggressive B cell lymphomas with a *MYC* breakpoint was observed.¹⁵ Based on this data in Burkitt lymphoma and DLBCL, it now seems justifiable to regard the presence of a complex karyotype with a t(8;14) as a diagnostic criteria for distinguishing Burkitt Lymphoma from Diffuse Large B-cell Lymphoma. The importance of this distinction argues for the retention of techniques that provide a genome wide survey of genetic abnormalities lymphoma.

An intrinsic problem with conventional cytogenetic analysis is that the resolution offered by banding techniques is low at approximately 4-5 Mb (Table 1). This means that structural alterations like translocation breakpoints may look similar at the light microscopic level but are different at the molecular level. For instance approximately 15% of all myelomas contain a t(11;14)(q13;q32) which appears identical to the characteristic translocation seen in mantle cell lymphoma. However, at the molecular level the breakpoint in myeloma affects the *IGH* locus at switch regions instead of the VDJ complex in mantle cell lymphoma and thus imply a different cellular origin.^{18, 19}

In spite of these limitations conventional cytogenetics remains a useful method to detect both specific primary and secondary abnormalities and an overall assessment of genetic complexity in lymphomas. It remains a valuable method to find new recurrent abnormalities and the systematic collection of data in the Mitelman database, which in July 2010 contained karyotypic data of 7583 mature B cell and 1265 mature T cell lymphoma cases, is a highly valuable source for data-mining (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>).

2. Multicolour fluorescent in situ hybridization (FISH) on chromosomes.

A ability of conventional metaphase cytogenetics to detect genome-wide genetic changes has been expanded by the introduction of multicolour painting of chromosomal preparations, by multiplex FISH, spectral karyotyping or COBRA FISH (combined binary ratio labelling

FISH).²⁰⁻²⁴ In these methods multiple labels (up to > 50) are generated by using mixtures of different fluorochromes. These labels are used to generate probes to multiple genomic regions including individual chromosomes or long and short chromosomal arms. are labelled with such mixtures. These labelled genomic sequences are subsequently hybridized to a metaphase chromosome preparations. Complex structural abnormalities can be visualized readily and the chromosomal origin of abnormal structures like marker chromosomes can be identified much more easily than by conventional cytogenetics. However, these methods require specific interpretative skills and more complex equipment than conventional cytogenetics, and therefore are only accessible to limited number of investigators.

3. Conventional comparative genomic hybridization (CGH) and array comparative genomic hybridization (aCGH)

Genome wide methods that do not need dividing tumour cells are comparative genetic hybridization (CGH)²⁵⁻²⁷ and its successor array CGH (aCGH). The principles of both methods are shown in Figure 1. A major drawback of both techniques is that balanced chromosomal alterations like translocations cannot be detected. Conventional CGH is based on a competitive hybridization between normal (for instance Cy3-labeled) DNA and tumour cell derived (for instance Cy5-labeled) DNA, where chromosomes obtained from normal individuals are used as a template for hybridisation. Thus the availability and quality of normal chromosome preparations may be a limiting factor. Moreover, the resolution is not much better than of conventional cytogenetics (Table 1).

Conventional CGH has been replaced by array CGH or “matrix CGH” in which the DNA fragments used for competitive hybridization are spotted on a glass slide and not on normal chromosomes. Depending on the number of (overlapping) probes used to create the platform, aCGH has a very high resolution at less than 50-100 kb. DNA isolated from paraffin tissue blocks can be successfully used for hybridization. Up to now approximately 65 publications have appeared on the application of array CGH in mature B cell and T cell lymphomas.²⁸⁻³⁴ and these show distinct patterns of gains and losses characteristic of particular lymphoma subtypes. Within the group of DLBCL, activated B cell (ABC) type DLBCL have a different pattern from germinal centre B cell (GCB) type DLBCL, with recurrent trisomy 3, gain of 18q and 19q, as well as loss of 6q in ABC type and gain/amplification of 2p and loss of 1p and 13q in GCB type of DLBCL as well as frequent gain/amplification of 9p in primary

mediastinal type of lymphomas (Table 2).³⁵ Similarly, distinct features were found between different types of T cell lymphomas.

4. Single nucleotide polymorphism (SNP) analysis.

SNP analysis is the most recently introduced whole genome method and has the potential advantage of an even higher resolution.^{36,37} Figure 2 illustrates the analysis of a series of paediatric BL using either aCGH (n=15; figure 2A) or SNP analysis (n=18; figure 2B) (Kluin et al, unpublished data). The higher resolution with SNP analysis is also shown in Figure 2C, in which we encountered a homozygous deletion at chromosome 9p21 affecting CDKN4 and CDKN6, two important tumour suppressor genes, that was missed by both conventional and array based CGH analysis. A unique advantage of SNP analysis over all other methods is that not only changes in copy number but also regions of loss of heterozygosity without loss or gain of DNA can be detected, indicating that both alleles are homozygous and derived from one parent. This loss of heterozygosity without changes in copy numbers is called uniparental disomy (UPD).³⁸⁻⁴² UPD may be important to detect since regions of UPD may harbour one or more genes with inactivating mutations (classical tumour suppressor genes). Since in a region with UPD both alleles will have the mutation, the Knudson two hit rule for tumour suppressor genes is fulfilled. Alternatively, regions with UPD may contain bi-allelic hypermethylated and thus silenced (candidate tumour suppressor) genes. SNP analysis is a promising approach to genome wide analysis in lymphoma and is now supported by efficient commercially available platforms which are widely available.

The major problem of SNP analysis is the difficulty in distinguishing acquired abnormalities from polymorphisms. This can only be conclusively resolved by analysing paired germ line (for instance derived from a buccal swab) and tumour material from the same patient.³⁶ Comparing the data from the individual cancer with large SNP databases of germ line material to correct for all potential polymorphisms is still not reliable and this analysis often results in the exclusion of many regions smaller than 250kb. Polymorphic regions, can even span several megabases, indicating that such a threshold may be even insufficient. Therefore, at the present time, except for the detection of UPD, SNP analysis on archival materials where germline DNA is not available is not superior to aCGH for the genome wide assessment of structural abnormalities.

CGH, aCGH and SNP analysis all have the intrinsic disadvantage that balanced genetic abnormalities (abnormalities without any gain or loss of DNA), and thus most translocations and inversions are missed (Table 1). Interestingly, due to the fact that many structural abnormalities like chromosomal breakpoints in lymphomas are associated with mutations and also small deletions or duplications of several nucleotides, such abnormalities may in fact be detectable by SNP analysis. However, this potential of SNP analysis has not been systematically addressed so far.

5. Interphase (and metaphase) fluorescent in situ hybridization

Interphase fluorescent in situ hybridization (interphase FISH) on cytospin preparations, imprinted fresh cells or paraffin tissue sections or metaphase cells of the tumour (metaphase FISH) has become a routine technique in many laboratories.^{11, 12, 43-47} The technique is mainly used to detect well characterised structural chromosomal abnormalities such as recurrent chromosomal breakpoints or juxtaposition of two different loci involved in chromosomal translocations. The method can also be used to detect amplification or low level gains and losses of loci. However, real amplifications as seen in solid tumours are relatively rare in lymphomas. Moreover, the detection of low copy gains and losses in tissue sections is much more problematic than detection of breakpoints for which sets of probes are available that allow easy “pattern recognition” (see below).⁴⁸ Identification and counting of signals per cell on tissue sections is impeded by many factors like cutting artefacts and nuclear overlap related to the thickness and homogeneity of the tissue sections, the size of the nuclei, as well as the fixation. Thus for a reliable assessment of subtle copy number variations (low levels of gains and losses) FISH on isolated nuclei either prepared from intact cell suspension or nuclear suspensions from paraffin blocks is preferable.⁴⁹⁻⁵²

For detection of the most common recurrent translocations including those affecting 8q24/*MYC*, 18q21/*BCL2*, 3q27/*BCL6*, 18q21 / *MALT1* efficient probe sets and protocols for hybridization procedures are now commercially available. These are based on two colour probe sets which generate patterns that allow normal cells to be distinguished from those with a mono-allelic or bi-allelic breakpoint.^{12, 48}

A good example of the usefulness of interphase FISH is detection of 8q24/*MYC* breakpoints and exclusion of other breakpoints in Burkitt lymphoma. In >90% of all Burkitt lymphomas a t(8;14)(q24;q32), t(2;8) or t(8;22) will be found whereas t(14;18)(q32;q21) affecting *BCL2*

and 3q27 breakpoints affecting *BCL6* should be absent. In contrast in 5-20% of all diffuse large B-cell lymphoma *MYC* breakpoints can also be detected, more than half of these cases sharing a t(14;18)(q32;q21) and / or a 3q27 breakpoint, thus representing a so called “double hit lymphoma”.¹ The latter lymphomas were previously diagnosed as diffuse large B cell lymphoma, Burkitt-like lymphoma, atypical Burkitt lymphoma or aggressive B cell lymphoma NOS and represent very aggressive lymphomas that behave differently from classical Burkitt lymphoma and are difficult to treat with regimens like R-CHOP or high intensity – short duration polychemotherapy.^{5, 53}

MYC breakpoints can be detected by FISH in different ways. The most widely used approach is a segregation or split assay using two differently coloured probes that flank the *MYC* locus at 8q24. In case of a green and red label, the two probes are seen as a yellow or red/green signal in normal interphase nuclei. In case of a mono-allelic break, the nucleus will show one normal signal derived from the normal 8q24 allele and one split signal with separate green and red signals. It is estimated that more than 90% of the breakpoints in Burkitt lymphoma are detected by the currently available probe sets. The few failures are partly due to the presence of cryptic rearrangements that can be missed by some assays.^{43, 54} In this *MYC* segregation assay, only a *MYC* breakpoint is detected and no information is given on the partner that is juxtaposed to *MYC*. In Burkitt lymphoma the partners are exclusively immunoglobulin loci (85% *IGH*, >10% lambda and <5% kappa), so for the diagnosis of Burkitt lymphoma the segregation assay could be replaced by more specific co-localization assays for *MYC* with *IGH*, or *MYC* with *IGL* and *MYC* with *IGK*. However, in other lymphomas like diffuse large B-cell lymphoma with a *MYC* breakpoint, *IGH/IGK/IGL* and *MYC* are juxtaposed in only two thirds of the cases. In the other cases loci like a locus at 9p13 or *BCL6* at 3q27 are the partners of *MYC*. Thus, to detect all 8q24/*MYC* breakpoints in Burkitt lymphoma and diffuse large B-cell lymphoma (and other lymphomas), all cases should always first be assessed by a simple *MYC* segregation assay. After this screening, a FISH test for juxtaposition of the *MYC* locus to one of the immunoglobulin loci should be applied, since absence of any type of co-localization with the immunoglobulin genes is strong evidence against a diagnosis of Burkitt lymphoma.¹³ **Figure 3 shows an example of a *MYC*-*IGH* co-localisation FISH pattern in a case of Burkitt lymphoma with a *MYC*-break.**

Because of the diagnostic and therapeutic consequences it is recommended that 8q24/*MYC* FISH analysis is carried out on all B cell lymphomas that share some features with Burkitt

lymphoma, particularly in adult patients. The analysis should include a segregation assay for 8q24/ *MYC*, and preferably also a co-localization assay for *MYC* and the *IG* loci, as well as a segregation assay for both *BCL2* and *BCL6* to rule out a double hit lymphoma.

One major drawback of FISH tests is the need to have immunofluorescence microscopy equipment and expertise to analyse the slides in a dark room. It sometimes is difficult to find the optimal regions with most tumour cells in such slides. Several companies are currently preparing bright field **in situ hybridization methods** (BRISH) making use of chromogenic substances **and/or silver** that could replace **dual colour** FISH tests, allowing pathologists to read slides at the normal bright-field microscope and to better correlate the signal pattern with the microscopic anatomy of the tumour.⁵⁵⁻⁵⁷ An additional advantage of BRISH over FISH is that the specimen can be archived and that inter-laboratory quality control programs are much easier to perform. **However, the major problem of BRISH is the visualization of exactly colocalizing signals.**

6. Polymerase chain reaction (PCR), real time polymerase chain reaction PCR (RT-PCR).

In the diagnosis of lymphoma PCR tests at the DNA level are used mostly for clonality analysis of the immunoglobulin genes or T cell receptor genes. Highly sensitive and specific primer combinations and protocols have been developed most notably by the BIOMED consortium (<http://www.euroclonality.org/>). These tests are currently used worldwide and enable to detect clonality in fresh tissues as well as in archival, **FFPE** materials.⁵⁸⁻⁶¹

Some translocations, such as the t(14;18) involving *BCL2*, can be detected at the genomic level by PCR. However in view of the diversity of breakpoints multiple tube tests with many sets of different primers are necessary to detect most t(14;18) breakpoints.⁶¹ This problem in detecting chromosomal breakpoints is even more evident for the t(11;14)(q13;q32) in mantle cell lymphoma. Extensive mapping studies have shown that only 30-40% of all t(11;14) breakpoints cluster in a small region (MTC) in which the breakpoint can be detected by PCR.^{62 63} Since FISH probes that cover all breakpoints can be easily designed, this technique is superior. Another disadvantage of PCR is that **this method** in general makes use of primers that flank the breakpoint, and therefore the primers have to be derived from both partners. In consequence both partners should be consistently involved, otherwise breakpoints will be missed.

On the other hand, if a breakpoint is amplifiable by PCR, it is a far superior method for the detection and quantification of small numbers of cells, and thus in the detection of minimal residual disease (MRD). PCR has been used to detect MRD after intensive chemotherapy and stem cell transplant in patients with mantle cell lymphoma with an amplifiable t(11;14) breakpoint.⁶⁴

Quantitative RT-PCR is very useful for the detection of chromosomal translocations that lead to the production of novel fusion mRNA transcripts. Such events form the minority of translocations in lymphomas (but the majority in myeloid neoplasms), since most translocations are derived from RAG1/2 or AICDA mediated juxtaposition of oncogenes to the enhancers of immunoglobulin gene or T cell receptor genes, leaving the protein-encoding domains intact. Examples of translocation that generate fusion transcripts and proteins are those involving ALK1 in ALK+ anaplastic large cell lymphomas (ALK+ ALCL) and ALK+ large B cell lymphoma (ALK+ DLBCL). In the great majority ALK and NPM are fused giving rise to a fusion protein with nuclear and cytoplasmic localization. In addition in 5-30% of the gastric and in 30-50% of pulmonary marginal zone B cell (MALT) lymphomas, a t(11;18)(q21;21) with fusion of API2 and MALTI is present. In gastric lymphomas the presence of this translocation is associated with resistance to Helicobacter pylori eradication therapy. Because of the therapeutic consequences it is recommended to either FISH or RT-PCR to detect this translocation routinely in marginal zone lymphomas.⁶⁵

The better performance of RT-PCR on cDNA than PCR on DNA in the detection of translocations is because the number of exons involved in the breakpoints is limited and RNA splicing eliminates long stretches of (intronic) nucleotides that can not be efficiently amplified by PCR on DNA. In consequence, most fusion breakpoints can be detected by RT-PCR with a very limited number of primer combinations. Also because of these characteristics, RT-PCR can be used on FFPE material. Formalin fixation has the advantage that endonucleases that degrade RNA are rapidly inactivated. On the other hand, apart from cross-linking and fragmentation of RNA, formalin also induces the addition of monoethylol groups to adenosine and consequently inhibits primer annealing for reverse transcription. Specific protocols are now for optimal RNA extraction and efficient cDNA synthesis from FFPE material.

Despite all optimized procedures, the efficiency of RT-PCR using RNA extracted from FFPE material remains approximately 30-50-fold lower than RNA extracted from matched frozen materials. This topic has been extensively reviewed by Farragher et al and the reader should consult these sources for more detailed information on the protocols that are now available ⁶⁶⁻

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7. Other molecular methods.

Several other molecular methods can be used for quantification of DNA targets and thus for the detection of copy number variations. They include quantitative fluorescent PCR of short tandem repeats (QF-PCR of STR), multiplex PCR of short exonic sequences (QMPSF) and multiplex ligation probe amplification (MLPA).⁷⁰ In principle all techniques enable to assay multiple loci for gains or losses, and therefore they could be helpful in the assessment of the genomic complexity, for instance as a tool to differentiate between Burkitt lymphoma and diffuse large B cell lymphoma. As far as known, none of these techniques have been introduced yet into a diagnostic haematopathology setting. Moreover, like for CGH methods and SNP analysis, these methods do not allow detecting reciprocal translocations.

8. Immunohistochemistry and molecular cytogenetics.

This review would not be complete without a comment on the use immunohistochemistry for the detection of genetic abnormalities. In fact immunohistochemistry may in some instances replace molecular cytogenetics.⁷³ Chromosomal abnormalities cause deregulation of genes that are not expressed in normal B cells or T cells, or are not expressed in a specific stage of maturation of normal B or T cells. An example is the t(11;14)(q13;q32) present in >95% of all mantle cell lymphomas. This translocation gives rise to activation of *CCND1* by juxtaposing to an immunoglobulin gene enhancer, and in consequence *CCND1* expression is induced while it is not expressed in normal B and T cells. Previous work has shown that there is an excellent correlation between the presence of a translocation and expression of *CCND1* at the RNA and protein level (western blot and immunohistochemistry).^{63, 74} Excellent monoclonal antibodies against *CCND1* have been developed and in consequence immunohistochemistry for *CCND1* has almost completely replaced the molecular methods to detect this translocation.⁷⁵

A **second** example is the detection of *ALK* translocations in *ALK*+ anaplastic large cell lymphoma (ALCL) and *ALK*+ diffuse large B cell lymphoma. These lymphomas and also a subset of inflammatory myofibroblastic tumours contain a fusion of the *ALK* gene at 2p23 to a large variety of other partners (*NPM* being the most frequent partner). Since *ALK* is not expressed in normal adult tissues, *ALK* expression in these tumours as observed by immunohistochemistry shows that a fusion protein containing *ALK* is present (table 2). In all lymphomas and inflammatory myofibroblastic tumours with an *ALK* breakpoint this expression is high and can be easily detected by immunohistochemistry. In consequence, immunohistochemistry has completely replaced molecular methods. Notably, the pattern of its expression within the cells as seen in tissue slides informs us also on the *ALK* fusion partner used in the translocation.⁷⁶ Recent research has shown that *ALK* inversions involving the *EML4* gene occur in approximately 4-9% of all adenocarcinomas of the lung. However, in these cases a very low level of *ALK* protein expression is present that only can be detected using specific antibodies and very sensitive assays.⁷⁷

A third example is the use of antibodies against *SOX11* in the diagnosis of mantle cell lymphoma. *SOX11* encodes a member of the *SOX* (*SRY*-related HMG-box) family of transcription factors involved in the regulation of embryonic development. Based on gene expression studies this gene appeared to be highly expressed in almost all mantle cell lymphomas in addition to some acute lymphoblastic lymphomas / leukaemias, Burkitt lymphomas and hairy cell leukaemias. Interestingly, *t*(11;14) and *CCND1* protein negative mantle cell lymphomas are *SOX11* positive.⁷⁸⁻⁸⁰ Other publications suggest that very indolent but also rare very aggressive mantle cell lymphomas may be negative for *SOX11*.^{81, 82}

Molecular cytogenetic aspects of individual lymphomas

In Table 2, a brief summary is given of the most common molecular abnormalities in specific lymphomas,¹ the methods to detect these abnormalities, as well as their diagnostic and clinical relevance. Excellent web resources include (<http://atlasgeneticsoncology.org/>), <http://www.progenetix.net/> and <http://www.ncbi.nlm.nih.gov/sites/entrez?db=cancerchromosomes>) and elsewhere.^{4, 11}

Conclusions and future perspectives

Molecular genetics has now become an integral part of routine lymphoma diagnostics. In many laboratories, PCR based clonality tests and FISH investigations for specific chromosomal abnormalities are performed on a routine basis. One challenge for the future will be to develop multicolour BRISH instead of fluorescent in situ hybridization (FISH) assays for detection of all breakpoints and relevant numerical abnormalities in lymphomas. The introduction of reliable BRISH assays will allow many more pathologists to perform and interpret these tests.

High throughput methods such as array CGH, SNP analysis and whole genome expression analysis have not been introduced into routine diagnostic laboratories. This may be facilitated in the near future by providing tailor made platforms with a limited number of targets to be detected. There is clearly a place for such assays, for instance for the differential diagnosis between Burkitt lymphoma and diffuse large B cell lymphoma. Similar approaches could likely be followed to distinguish indolent from conventional mantle cell lymphomas, since there are indications that indolent mantle cell lymphomas have a relatively simple karyotype.⁸² At present, these assays require fresh or freshly frozen material and the question whether these methods will become part of our routine diagnostics is very much dependent on the question whether these techniques will be applicable in routinely FFPE materials.

Finally, it is obvious that the development of new drugs that target specific intracellular or intercellular pathways in specific types of lymphoma, will drive the development of new molecular tests in malignant lymphoma. One example will be new drugs that interfere with specific signalling pathways used in ABC type diffuse large B-cell lymphoma but not GC type.⁸³ This will require better methods to distinguish these types of diffuse large B-cell lymphoma, and the detection of the specific molecular abnormalities in the pathway targeted by the drug. Developments of this type are already taking place in other types of tumour.

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Legends to the Figures

Figure 1. Principles of conventional CGH and array CGH.

Normal DNA and tumor DNA are differentially labeled, often with Cy3 and Cy5. After blocking of (ubiquitously present and thus hindering) repetitive DNA, these labeled DNAs are competitively hybridized to either normal chromosome preparations or a high density array of (preferably) overlapping probes on a glass slide. In case of conventional CGH, the preparations can be directly analyzed on a fluorescence microscope, in cases of an array, dedicated hardware (a microarray scanner) and software is necessary.

Figure 2. Array CGH analysis and SNP analysis of paediatric Burkitt lymphoma.

Panel A shows conventional CGH analysis of a series of 15 paediatric Burkitt lymphomas is shown. Each black bar to the right of a chromosome represents gain in an individual case; each grey bar to the left side of a chromosome represents loss. This analysis shows a limited number of recurrent aberrations, such as copy number increase of chromosome 13q31-32 and loss of chromosome 17p.

Panel B shows a SNP analysis of 18 paediatric Burkitt lymphomas is shown. The human CNV370-DUO BeadChips (Illumina Inc, San Diego, CA, USA) array was used. SNP array data were imported into BeadStudio (Illumina Inc, San Diego, CA, USA) and analyzed using the Chromosome bookmark plug-in and a modified version of SOMATICS. Directly to the right of each chromosome, the black bar indicates the region that is covered by the probes. Gains are indicated by green bars right of the chromosomes, losses by red bars (left of the chromosome). Note the higher resolution but also the higher complexity, with in particular a larger number of smaller events, in particular around the centromeres of chromosomes. A large number of these alterations reflect copy number variations between individuals and are not tumour specific.

Panel C shows a case analyzed by SNP's with a small homozygous deletion on 9p21, containing only the CDKN2A and CDKN2B genes. In a second case (not shown) there was a large hemizygous deletion and a similar small deletion on the other allele. These small deletions were not detected by either conventional CGH or array CGH (data not shown).

Figure 3. Colocalization of MYC and IGH in Burkitt lymphoma.

A case of paediatric Burkitt lymphoma with presentation in the tonsil with a MYC-break (detected with the Vysis LSI MYC Dual Color, Break Apart Rearrangement Probe set from Abbott molecular; data not shown), was hybridized with the Vysis LSI IGH/MYC, CEP 8 Tricolor, Dual Fusion Translocation Probe. Many cells show fusion of the MYC (green) and IGH (red) signals (arrows). Note that the percentage of spurious co-localizing signals can be high in normal cells, up to 20% of all cells.

For Peer Review

TABLE 1. INTRINSIC FEATURES OF MOLECULAR GENETIC METHODS

Method	Resolution	Possible on formalin fixed materials	Levels of required skills & equipment	Individual locus unique probes / primers required for the assay	Ability to detect:					
					genome-wide structural abnormalities	genome-wide numerical abnormalities	Specific translocations	Specific amplifications	Low copy number changes	UPD
karyotyping	>4-5 mb	-	++	-	++	+	+	±	±	-
Spectral karyotyping	>1-5 mb	-	+++	-	+++	+	+	±	±	-
CGH	>5mb	+	++	-	-	++	-	±	+	-
Array CGH	>50kb	+	+++	-	-	+++	-	++	++	-
SNP array analysis	>50kb ^a	+	+++	-	-	+++	-	++	++	++
Interphase FISH (sections)	>50kb	+	+	+	-	-	++	++	+	-
PCR	<400bp ^b	+	+	+	-	-	± ^c	++	-	-
RT-PCR	<150bp ^d	+	+	+	-	-	±	-	-	-

UPD: uniparental disomy; CGH: comparative genomic hybridization; SNP: single nucleotide polymorphism; FISH: fluorescence in situ hybridization; PCR: polymerase chain reaction; RT-PCR: reverse transcription PCR. ^a: Theoretically very high resolution, practically limited by polymorphisms. ^b: <400 bp in formalin fixed materials. ^c: very much dependent on the individual translocations, see text. ^d: in formalin fixed materials; only for fusion genes, see text

TABLE 2. INVENTORY OF MOST COMMON GENETIC ABNORMALITIES AND THEIR DIAGNOSTIC AND CLINICAL SIGNIFICANCE IN PERIPHERAL B- AND T CELL LYMPHOMAS*

Lymphoma	Primary event	frequency	Preferable method	comments
B cell lymphomas				
Mantle cell lymphoma	t(11;14)(q13;q32), <i>CCND1</i> & <i>IGH</i>	>95%	Immunohistochemistry (CCND1); FISH; karyotyping	Except for the major translocation cluster, breakpoints at 11q13 are scattered over large region, PCR not useful t(11;14) is absent in extremely rare cases; diagnosis can only be made if all other features are consistent with MCL. Cases with simple karyotype and only the t(11;14) may have favourable prognosis
	Del 13q14, ?	40-50%	FISH; karyotyping; aCGH	
	Del 7p13, <i>TP53</i>	20-45%	FISH; karyotyping; aCGH	Associated with prolymphocytic morphology?
	Del 11q23, <i>ATM</i>	20-60%	aCGH; FISH, karyotyping	<i>ATM</i> mutations in 40-75%
	Del 9p21, <i>CDKN2A/INK4a</i>	20-30%	FISH; karyotyping; aCGH	Often small homozygous deletions
	tetraploidy	In 80% of pleomorphic and 35% of blastoid variants	karyotyping; aCGH	Associated with poor prognosis
	+8q24 and t(8)(q24), <i>MYC</i>	15-35%; 8q24 break in 5-10%;	FISH; karyotyping	Blastoid morphology, high Ki67, aggressive course

Follicular lymphoma	t(14;18)(q32;q21) <i>BCL2</i> & <i>IGH</i>	Grade 1/2: 90% Grade 3a: ? Grade 3b: 30% or lower	Immunohistochemistry (BCL2); FISH; karyotyping	Most breakpoints can be detected by multiple tube PCR		
	t(3)(q27), <i>BCL6</i> ; breakpoint at alternative breakpoint region (ABR) or major breakpoint region (MBR)	grade 3b: 30%	FISH; karyotyping	ABR is 270kb upstream of MBR	Some <i>BCL6</i> FISH tests miss ABR breakpoints	
Diffuse follicle centre cell lymphoma	t(14;18)(q32;q21) <i>BCL2</i> & <i>IGH</i>	100% (?)	FISH; karyotyping	According to the WHO classification this diagnosis can only be made after demonstration of the t(14;18)	However, some publications describe similar lymphomas without the translocation.	
Burkitt lymphoma	t(8;14)(q24;q32), <i>MYC</i> & <i>IGH</i> ; t(8;22)(q24;q11), <i>MYC</i> & <i>IGL</i> ; t(2;8)(p12;q24), <i>MYC</i> & <i>IGK</i>	>90%	FISH; karyotyping	Breakpoints may be far from <i>MYC</i>	Always co-localization with IG loci: FISH assays for 8q24 split and <i>MYC-IG</i> fusion recommended	
	Simple karyotype; alterations at 1q; +8; +12 or alterations at 13q3; mir17-92	<20%	aCGH, karyotyping, FISH	In 30-40% of BL the translocation is the only alteration (point mutations not included)	High genomic complexity likely excludes DX of BL	Combination with other translocations involving <i>BCL2</i> , <i>CCND1</i> (<i>BCL6</i> ?) exclude BL
Diffuse large B cell lymphoma NOS	t(3)(q27); <i>BCL6</i>	30-40%	FISH; karyotyping	karyotype may show very complex abnormalities and multiple subclones		<i>BCL6</i> mutations in majority of cases
	t(8)(q24), <i>MYC</i>	5-20%	FISH; karyotyping	In 40% <i>MYC</i> is not juxtaposed to IG locus but to a variety of other partners	>50% are double hit lymphomas and likely should be diagnosed as B cell	Associated with poor survival

						lymphoma unclassifiable
Specific in ABC type NOS	Del 6q; del 9p21, <i>CDKN2A</i> ; trisomy 3, <i>FOXP1</i> ?; +18q21, <i>BCL2</i> / <i>NFATC1</i> ; +19q, <i>SPIB</i>	15-35%	aCGH; FISH	Trisomy 3 and del 9p21 associated with poor survival	Subtype distinguished by IHC algorithm ⁷¹	
Specific in GCB type NOS	t(14;18)(q32;q21), <i>BCL2</i> & <i>IGH</i>		FISH; karyotyping	Associated with CD10 and BCL2 protein expression	Subtype distinguished by IHC algorithm ⁷¹	
	Del 1p, <i>TP73</i> ; amp 2p, <i>REL</i>		aCGH; FISH			
Primary mediastinal large B cell lymphoma	+ 9p, <i>JAK2</i> / <i>PDCD1LG2</i> ; amp 2p, <i>REL</i>	15-40%	aCGH; FISH			
Primary DLBCL leg type	Similar to ABC type DLBCL NOS; t(3)(q27), <i>BCL6</i> ; t(8)(q24) or gain, <i>MYC</i> ; +18q21 or amplification; del9p21, <i>CDKN2A</i>	30->60%	aCGH; FISH		Diagnosis made by combination of features (clinic, morphology, phenotype)	
DLBCL of the CNS	Similar to ABC type DLBCL NOS; t(3)(q27), <i>BCL6</i> ; +18q21 or amplification; del9p21 <i>CDKN2A</i> ; del 6p21.3 (HLA)	See ABC type of DLBCL	aCGH, FISH	Small deletion in HLA region seem to be unique for DLBCL of the CNS, testis		
T cell/histiocyte rich large B cell lymphoma	No recurrent abnormalities	-	-			
ALK+ DLBCL	t(2;17)(p23;q23), fusion of <i>CLTR-ALK</i> ; rarely t(2;5)(p23;q35), fusion of <i>NPM-ALK</i>	100%	Immunohistochemistry (ALK), FISH; RT-PCR	Most cases are plasmablastic, CD20-, CD79a-, CD45-, CD138+, cyIg +	Poor prognosis; perhaps better in children	
Double hit lymphomas	t(8)(q24) & t(14;18)(q32;q21) or t(8;14;21)(q24;q32;q21)	60%	FISH; karyotyping	Mostly in elderly patients; at least half of all DLBCL with a <i>MYC</i> breakpoint are DH lymphoma	Aggressive behaviour, high tumour load, bone marrow and CNS localization & poor prognosis	

	t(8)(q24) & t(3)(q27) or t(3;8)(q27;q24)	<10%	FISH; karyotyping	Probably also poor prognosis
	t(8)(q24) & t(14;18)(q32;q21) & t(3)(q27)	15%	FISH; karyotyping	Also called "triple hit lymphoma" Aggressive behaviour, high tumour load, bone marrow and CNS localization & poor prognosis
CLL / small lymphocytic lymphoma	Del 13q14.3, miRNA 16-1 and 15-a ?	30-50%	FISH; aCGH	associated with favourable prognosis
	Del 11q22-23, <i>ATM</i>	10-20%	FISH; aCGH	
	Trisomy 12	10-20%	FISH; aCGH	associated with atypical CLL
	Del 17p13, <i>TP53</i>	10%	FISH; aCGH	associated with extremely poor prognosis
Splenic marginal zone lymphoma	Del 7q31-32, <i>CDK6</i> ?	40% ?	FISH; aCGH	no prognostic significance
MALT marginal zone lymphomas	t(11;18)(q21;q21), <i>API2</i> & <i>MALT1</i>	5-30% of gastric and 30-50% of pulmonary MALT lymphomas	RT-PCR, FISH	Associated with resistance to HP eradication therapy
	t(14;18)(q32;q21), <i>IGH</i> & <i>MALT1</i>	0-20% of ocular & salivary MALT lymphomas	FISH	
	t(1;14)(p22;q32), <i>BCL10</i> & <i>IGH</i>	<10% in all MALT lymphomas	FISH	
	Trisomy 3	Up to 50% in all MALT		

lymphomas				
Nodal marginal zone lymphoma	No specific abnormality (trisomies)	-	-	
Lymphoplasmacytic lymphoma	Del 6q	40-60% ?	FISH; aCGH	No prognostic significance
Hairy cell leukemia	No specific abnormality	-	-	
T-cell lymphomas				
T-cell prolymphocytic leukemia	t(14;14)(q11;q32) or inversion, <i>TCRA</i> & <i>TCL1B</i> ; t(X;14)(q28;q11), <i>MTCP1</i> & <i>TCRA</i>	80-100%	karyotyping	
	Abn 8p11 and t(8;8)(p11-12;q12),	80%	karyotyping	
	Del 11q23, <i>ATM</i>		Karyotyping; FISH	
T-cell large granular lymphocyte leukemia	No specific abnormality	-	-	
Extra nodal NK/T cell lymphoma, nasal type	Del(6)(q21q25) or i(6)(p10)		aCGH, karyotyping, metaphase FISH	
Enteropathy associated T cell lymphoma, type A	+9q31.3; del16q12; +1q31-q41; +5q34-q35	80%	aCGH	CD3+, CD8-, CD56-, CD30+ in >50%
Enteropathy associated T cell lymphoma, type B	+9q31.3; del16q12; +8q24; <i>MYC</i>	80%	aCGH	CD3+, CD8+, CD56+
Hepatosplenic T-cell lymphoma	Abn #7, incl ring chromosome 7		Karyotyping, metaphase FISH	
Angioimmunoblastic T cell lymphoma	Trisomy 3, 5		aCGH	
Peripheral T cell	Many imbalances incl gains at		aCGH	

lymphoma NOS	7q, 8q, 17q, losses at 4q, 5q, 6q				
Anaplastic large cell lymphoma ALK+	t(2;5)(p23;q35), fusion of <i>NPM</i> & <i>ALK</i> ; many other variant translocations involve <i>ALK</i> at 2p23 (fusion)	t(2;5) in 85%; variants in 15%	Immunohistochemistry for ALK; FISH, RT-PCR	IHC pattern can reveal fusion gene of <i>ALK</i> (e.g. <i>NPM</i> : nuclear and cytoplasmic; <i>CLTR</i> granular cytoplasmic)	Better prognosis than ALK negative ALCL
Anaplastic large cell lymphoma ALK-	No translocations involving <i>ALK</i> ; many imbalances		Immunohistochemistry for ALK; FISH, RT-PCR	Worse prognosis than ALK positive ALCL	

*: Rare disorders, cutaneous lymphomas, EBV, HTLV1 and HHV8 associated lymphoproliferative disorders excluded

blue: diagnosis that more or less is defined by its genetic abnormality (or the associated immunohistochemical marker); yellow: genetic abnormality is important prognostic abnormality in clinical setting

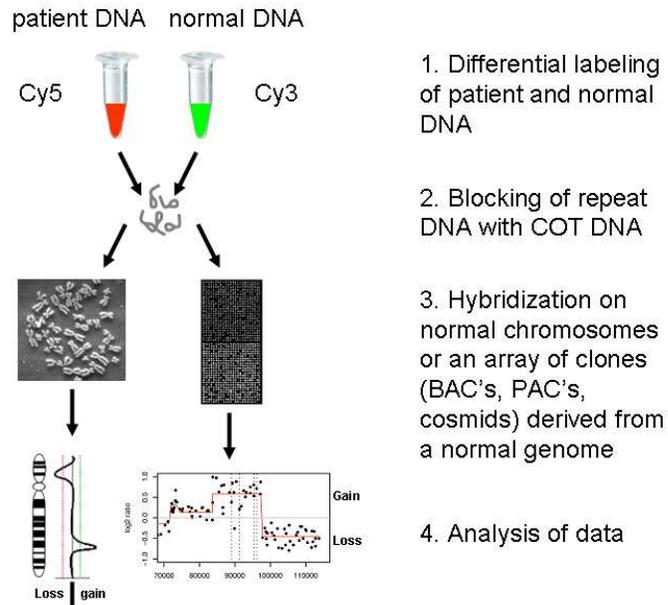


Figure 1. Principles of conventional CGH and array CGH.

Normal DNA and tumor DNA are differentially labeled, often with Cy3 and Cy5. After blocking of (ubiquitously present and thus hindering) repetitive DNA, these labeled DNAs are competitively hybridized to either normal chromosome preparations or a high density array of (preferably) overlapping probes on a glass slide. In case of conventional CGH, the preparations can be directly analyzed on a fluorescence microscope, in cases of an array, dedicated hardware (a microarray scanner) and software is necessary.

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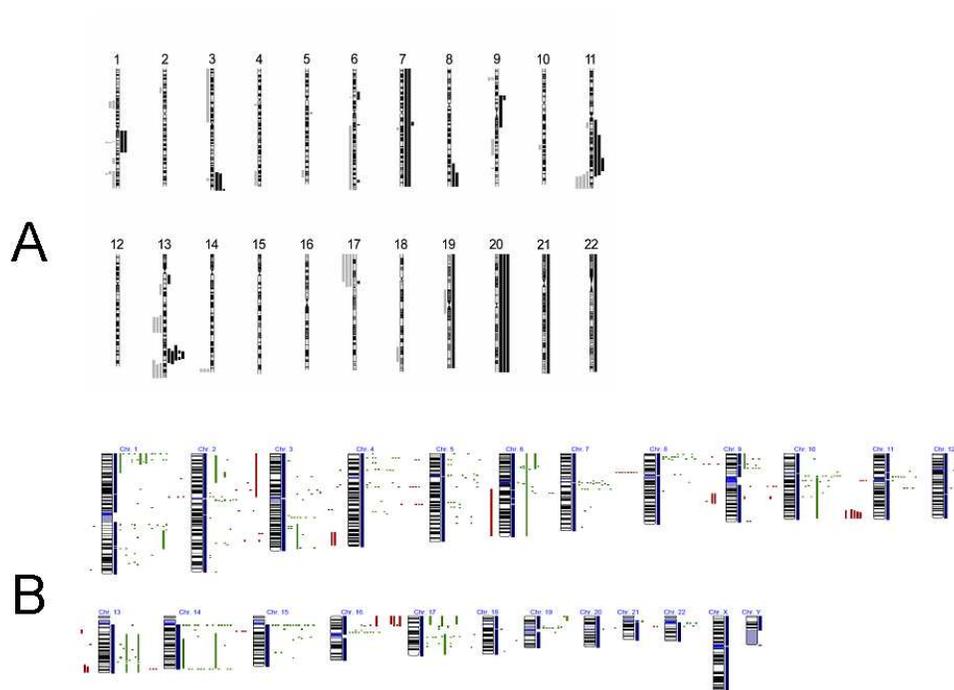


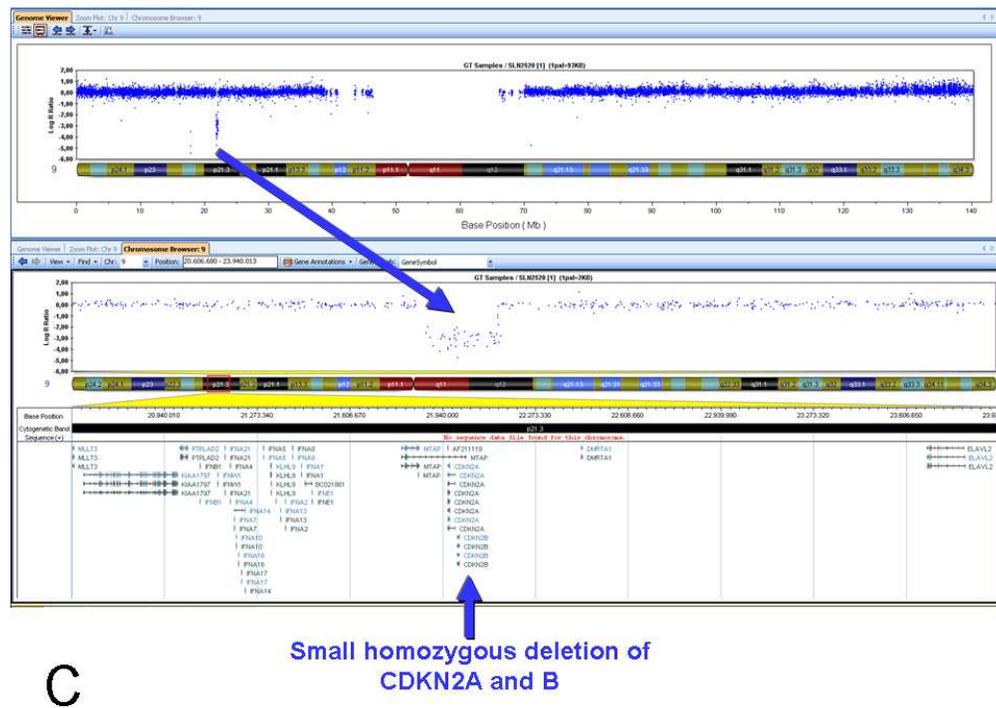
Figure 2. Array CGH analysis and SNP analysis of pediatric Burkitt lymphoma.

Panel A shows conventional CGH analysis of a series of 15 paediatric Burkitt lymphomas is show. Each black bar to the right of a chromosome represents gain in an individual case; each grey bar to the left side of a chromosome represents loss. This analysis shows a limited number of recurrent aberrations, such as copy number increase of chromosome 13q31-32 and loss of chromosome 17p.

Panel B shows a SNP analysis of 18 paediatric Burkitt lymphomas is shown. The human CNV370-DUO BeadChips (Illumina Inc, San Diego, CA, USA) array was used. SNP array data were imported into BeadStudio (Illumina Inc, San Diego, CA, USA) and analyzed using the Chromosome bookmark plug-in and a modified version of SOMATICS. Directly to the right of each chromosome, the black bar indicates the region that is covered by the probes. Gains are indicated by green bars right of the chromosomes, losses by red bars (left of the chromosome). Note the higher resolution but also the higher complexity, with in particular a larger number of smaller events, in particular around the centromeres of chromosomes. A large number of these alterations reflect copy number variations between individuals and are not tumour specific.

Panel C shows a case analyzed by SNP's with a small homozygous deletion on 9p21, containing only the CDKNBA and CDKN2B genes. In a second case (not shown) there was a large hemizygous deletion and a similar small deletion on the other allele. These small deletions were not detected by either conventional CGH or array CGH (data not shown).

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C

Small homozygous deletion of
CDKN2A and B

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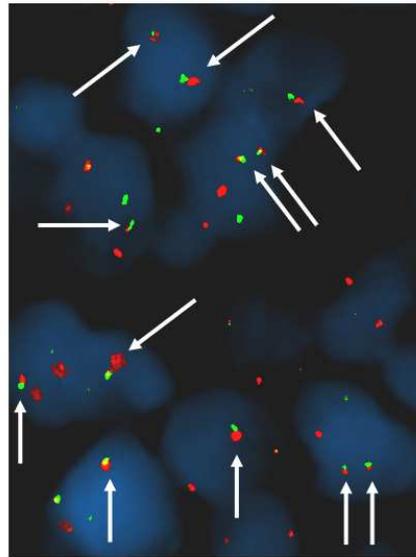


Figure 3. Colocalization of MYC and IGH in Burkitt lymphoma. A case of paediatric Burkitt lymphoma with presentation in the tonsil with a MYC-break (detected with the Vysis LSI MYC Dual Color, Break Apart Rearrangement Probe set from Abbott molecular; data not shown), was hybridized with the Vysis LSI IGH/MYC, CEP 8 Tri-color, Dual Fusion Translocation Probe. Many cells show fusion of the MYC (green) and IGH (red) signals (arrows). Note that the percentage of spurious co-localizing signals can be high in normal cells, up to 20% of all cells.

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