

# The CT-element of the *c-myc* gene does not predispose to chromosomal breakpoints in Burkitt's lymphoma

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**Abstract.** *Background:* Chromosomal translocations are causally related to the development of many tumors. In Burkitt's lymphoma, abnormalities involving the *c-myc* gene are essential. The CT-element of the *c-myc* promoter adopts non-B-conformation *in vivo* and *in vitro*, and therefore provides a potential fragile site. *Methods:* We have developed a LM-PCR-based approach to test if chromosomal breakpoints indeed cluster in this region. *Results:* Amplifying both, wild-type as well as the translocated *c-myc* gene by LM-PCR, it was shown that chromosomal breakpoints did not cluster within the CT-element. *Conclusions:* Therefore, the CT-element is not especially susceptible to the formation of breakpoints leading to chromosomal translocations in Burkitt's lymphoma.

**Keywords:** DNA-conformation, chromosomal breakpoints, *c-myc*, Burkitt's lymphoma, LM-PCR

## 1. Introduction

Chromosomal abnormalities, including reciprocal chromosomal translocations, are the hallmark of many hematological and solid cancers. These translocations often yield chimeric gene products or disturb the gene structure with subsequent activation of proto-oncogene expression. In both scenarios, transcription factors are frequently involved. Abnormal expression or function of regulatory proteins disturbs the action of downstream targets, eventually resulting in uncontrolled cell growth. Thus, the underlying translocations and genetic damage are responsible for the pathogenesis of cancer. In order to understand the molecular mechanisms leading to the formation of chromosomal breaks and/or the malfunction of the affected genes, it is of interest and importance to determine the exact sites of breakpoints.

Burkitt's lymphoma is one of the most extensively characterized malignancies caused by chromosomal

translocations [2]. Although several forms of translocations are observed in Burkitt's lymphoma, they all have in common the involvement of a region of chromosome 8 harboring the *c-myc* gene. After chromosomal translocation, the *c-myc* gene falls under the control of regulatory elements of one of the immunoglobulin genes. Subsequently, the expression of *c-myc* is dysregulated leading to the inappropriate activation or repression of *c-myc* downstream target genes with consequent oncogenic transformation.

Breakpoint cluster regions in the immediate flanking sequences of *c-myc* have been mapped by Southern blotting [4]. It is also known that a segment of the *c-myc* promoter close to the CT-element, a positive regulatory element 100–150 bp upstream of the *c-myc* P1-promoter, forms non-B DNA structure *in vitro*, and *in vivo* is reactive to single strand-specific chemicals [1,7]. There is compelling evidence that the DNA and chromatin structure is the primary determinant of the distribution of DNA damage [6]. The CT-element, therefore, could predispose to breakpoint formation, because of its ability to form a single stranded region, and, possibly, therefore, a fragile site. Testing of this hypothesis would require mapping of breakpoint locations at the sequence level.

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A local topology of single-stranded DNA has recently been shown to be responsible for breakpoint formation in case of the t(14;18)-translocation, associated with follicular lymphoma, involving the *bcl-2* gene [11].

On the chromosomal level, translocations can be detected and visualized by powerful cytogenetic methods. However, higher resolution mapping of breakpoints is traditionally performed by Southern blot hybridization. This approach has many limitations including the high amount of DNA needed, slow processing, and coarse resolution. Alternatively, fine mapping can be performed using direct sequencing, but this requires subcloning. PCR-based methods have been developed, but due to the diversity of the translocated immunoglobulin genes, these approaches usually require a set of multiple PCR reactions with complex combinations of *c-myc*- and immunoglobulin-specific primers [9].

To overcome these limitations and to test our hypothesis, we designed an alternative strategy, based on the utilization of ligation-mediated PCR [8]. Using this technique, the CT-element region was tested in order to determine whether translocations occurred directly in regions of non-B-DNA.

## 2. Materials and methods

### 2.1. Experimental design

The strategy used is outlined in Fig. 1. To examine the CT-element region of the *c-myc* gene, a set of three gene specific primers was selected from the sequence of the wild-type gene, according to the common criteria for LM-PCR [8]. Next, a restriction site upstream of the CT-element, i.e. the region of interest, was selected; for LM-PCR, the restriction site should generally not be too rare, and the distance from the gene specific primer should not exceed 400–500 bp. In this study, these criteria were fulfilled by a MwoI site. 11 kb of the *c-myc* locus around the CT-element contain 57 MwoI sites, averaging about one restriction site every 193 bases, with a cluster of three sites between nucleotides 190 and 250 upstream of the CT-element. Restriction analysis of the IgH locus shows a similar frequency of MwoI sites with one (statistic) cut every 218 bases.

Genomic DNA was digested with this restriction enzyme, primer extension with the first gene specific oligonucleotide was performed, and an adapter was

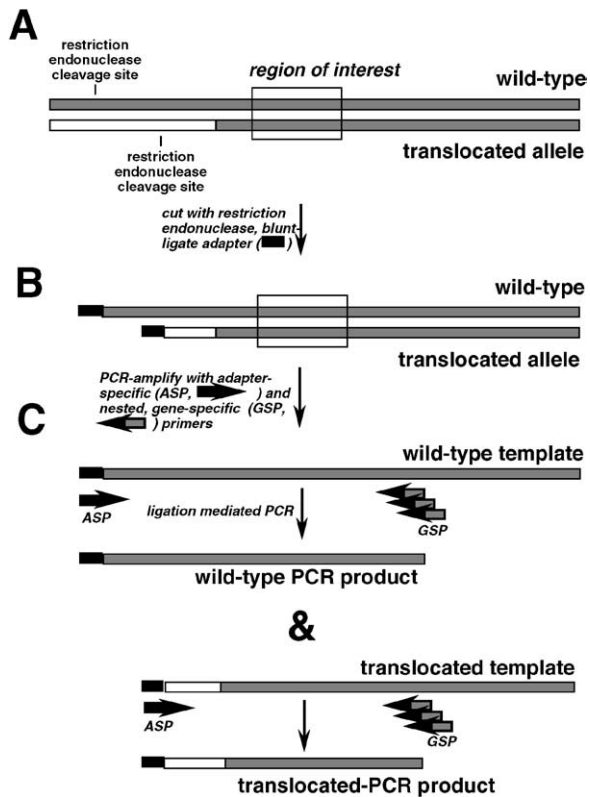


Fig. 1. Schematic outline of a LM-PCR based strategy for detection of chromosomal breakpoints. (A) Anatomy of the wild-type and translocated alleles around the region of interest. In the wild-type allele, a specific endonuclease restriction site is chosen within a defined distance upstream of the region of interest. In case of a translocated allele, this particular restriction site is lost, and a new specific site is located randomly either up- or downstream of the site of the wild-type gene. (B) After digestion with the corresponding restriction enzyme, the first gene specific oligo is annealed and a primer extension performed. Then, an amplification adapter is ligated (solid). (C) PCR amplification is performed with an adapter-specific primer (ASP) and a second gene specific primer (GSP). In case of an underlying translocation, this results in amplification of the wild-type gene and an additional PCR product of different size from the translocated allele.

ligated to the newly formed blunt-end. PCR amplification was performed using the adapter oligonucleotide and a nested, second gene specific primer. Using this approach a band diagnostic for the wild-type gene, and a band of different size for the translocated allele should be visualized.

We have applied this assay to a series of 9 Burkitt's lymphomas, previously shown by Southern blotting to harbor translocations in the vicinity of the region of interest. DNA isolated from primary Burkitt's lymphomas (available from previous studies with IRB approval) [4], or Burkitt's lymphoma cell lines by stan-

dard procedures. 4  $\mu$ g of DNA were then digested with MwoI, followed by phenol/chloroform purification.

## 2.2. Ligation-mediated PCR (LM-PCR)

A first gene specific primer composed of bottom strand sequence, 103 bp downstream of the CT-element, near the P1 start site, was annealed to denatured DNA, and a single extension reaction with Vent polymerase (New England Biolabs, Beverly, MA) was performed. The standard amplification adapter [8] was ligated overnight at room temperature. After additional phenol/chloroform purification, one half of the reaction was directly used as template, the other half was first digested with BssHIII – which should remove the adapter from the wild-type gene and so should suppress amplification of the un-rearranged allele. Following phenol/chloroform extraction, PCR-amplification was performed with the second nested gene-specific and upstream adapter-specific primers. A final cycle of PCR using a  $^{32}$ P-end-labeled, third gene specific primer was performed, and the products were displayed by electrophoresis on a 6% denaturing polyacrylamide gel and autoradiography. The primer sequences were previously described [7]. PCR conditions were as originally published [8]. To confirm the validity of our approach, PCR-products of two representative tumor samples were re-amplified by semi-

nested PCR using the adapter-specific as well a third gene-specific primer with Taq-Polymerase, separated by agarose gel electrophoresis, recovered from gel, and cloned into pGEM-T (Promega, Madison, WI). The cloned sequence was determined by automated sequencing using an ABI prism 377 according to the manufacturer's instructions, followed by a BLAST sequence analysis (<http://www.ncbi.nlm.nih.gov/BLAST>).

## 3. Results and discussion

The method outlined here was first tested on the extensively characterized Burkitt's lymphoma cell line Raji. This cell line is known to have a translocation of the *c-myc* gene outside the region of interest, but to harbor a 7-bp deletion within the region of interest in the translocated allele [10]. Using Raji DNA as template, we were able to amplify two bands of the expected size (Fig. 2).

Next, the study was extended to include 9 more samples from primary Burkitt's lymphoma and the expected PCR fragment of the *c-myc* gene was amplified in 8 out of these (Fig. 2). In 2 of these, only the wild-type band was amplified, indicate that no translocation in the region of interest existed. In contrast, 6 samples yielded an additional band of different size, indicating a translocated *c-myc* allele, or, as in case of Raji, an

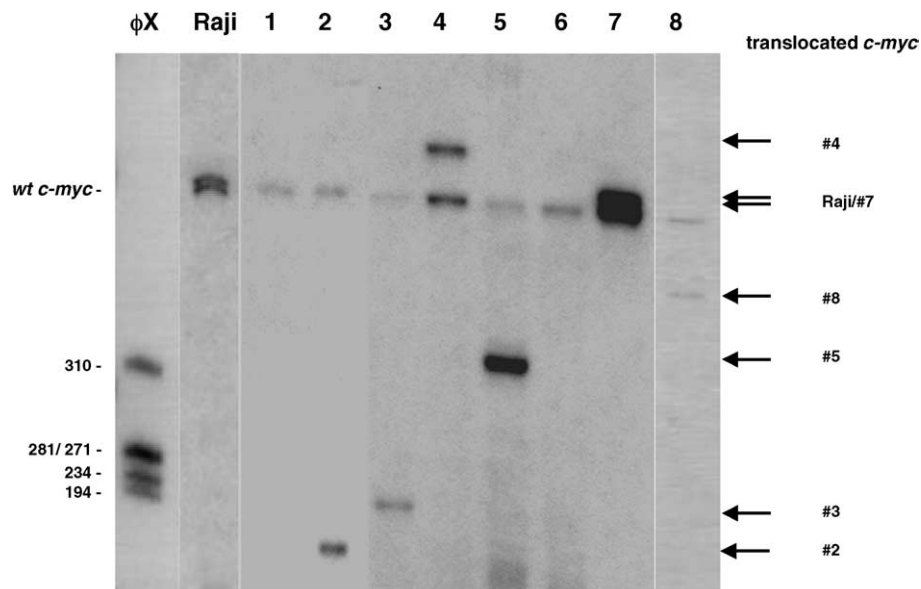


Fig. 2. LM-PCR based detection of *c-myc* translocations in Burkitt's lymphomas. Raji shows amplification of 2 distinct bands, due to a 7-bp-deletion in the region between the MwoI-site and the gene specific oligos. For tumors 2, 3, 4, 5, 7, and 8, a second band was amplified in addition to the 377-bp band of the wild-type *c-myc* allele, indicating a translocation in this region.  $\Phi$ x:  $\Phi$ x 174 FR DNA/Hae III digest marker.

otherwise altered gene. The translocated allele either led to amplification of a smaller (Fig. 2, lanes 2, 3, 5, and 8) or bigger (lanes 4 and 7) PCR product than the wild-type, depending on the sequence of the translocated gene and consequently the distribution of MwoI sites. These data for the first time show that an LM-PCR based approach is generally applicable for amplification of chromosomal breakpoints.

In addition, a modification of the procedure in which the amplification was performed subsequent to restriction enzyme digestion with BssHIII, allowed the selective amplification of the translocated allele. Cutting between the gene specific primer sequence and the adapter-ligated MwoI site suppressed amplification of the wild-type *c-myc* band, but not amplification of the translocated allele thus yielding only one PCR product (data not shown). This modification is likely to be especially important when performing LM-PCR from small amounts of tumor cells contaminated with non-neoplastic tissue.

To verify that the amplified bands originated from the wild-type as well as translocated alleles, the PCR products from tumors 2 and 7 were subcloned and sequenced. In both cases, sequence analysis confirmed that one of the two bands represented the wild-type *c-myc* gene, whereas the other had undergone a typical translocation to the IgM heavy chain locus at 14q32 [2] (Fig. 3).

Sequence analysis further revealed that the translocations in both tumors (2/2) analyzed did not occur immediately inside the CT-element, but at least 50 bp upstream thereof (Fig. 3). Together with the two cases in which LM-PCR did not provide a second band indicative of a translocation in relation to the CT-element, these data reveal little proclivity for this element to suffer inter-chromosomal recombination.

There are two findings emerging from this study. First, our results show that the CT-element of the *c-myc* gene, despite harboring a peculiar single-stranded DNA conformation, was not predisposed to strand breakage or illegitimate recombination in Burkitt's lymphoma. One could easily imagine that non-double stranded DNA, due to a higher vulnerability, might serve as a hotspot for breakage. The panel of Burkitt's lymphomas we have tested did not show that breakpoints occur precisely within the CT-element. However, since from Southern blot mapping results [4], translocation points are known to cluster at least in the vicinity of the CT-element, it remains speculative if the single-stranded structure of the CT-element might favor such events from a distance. Remarkably, a mecha-

#### tumor 2, lower band

```
GCGGTGACCCGGGAGATCTGAATTCCTGCCCCACCTGCCCC
TGCTCACCTGCGGCTGCTCTGCCCTGGTCCCTGAGCTCTCTT
ACTCTGTTTACATCCTAGAGCTAGAGTGCTCGGCTGCCGGCT
GAGTCTCCTCCCACTTCCCACTCCCACTCCCACTCCCACTCCCACT
AGGCCCTCCCGGTTCCCAAAGCAGAGGGCGTGGGGGAAAA
GAAAAAGATCCTCTCTCGCTAATCTCCGCCACCGGCCCTTT
ATAATGC
```

#### tumor 7, upper band

```
GCGGTGACCCGGGAGATCTGAATTCGGCTTCCATCTTTTGT
CTCCCTCTACTTGATACCCAGTTCCTCGAGTCTGCCCA
GCGCCACTGGGTTTTGGTTCCAAAGCATTACCAATCATTAC
TACCTCCACTACTGGGTGGAATATTTCTTTGCTGACCAAA
GGAAATGGTAGCGCGCTAGTTAATTCATGCGGCTCTCTTA
CTCTGTTTACATCCTAGAGCTAGAGTGCTCGGCTGCCGGCT
GAGTCTCCTCCCACTTCCCACTCCCACTCCCACTCCCACT
AAGCGCCCTCCCGGTTCCCAAAGCAGAGGGCGTGGGGGAA
AAGAAAAAGATCCTCTCTCGCTAATCTCCGCCACCGGCC
TTTATAATGC
```

#### tumor 7, lower band

```
GCGGTGACCCGGGAGATCTGAATTCGTCGCTTCTCTGTGTG
GAGGGCAGCTGTTCCGCTGCGATGATTTATACTCACAGGA
CAAGGATGCGGTTTGTCAAACAGTACTGCTACGGAGGAGCA
GCAGAGAAAGGAGAGGGTTTGAGAGGGAGCAAAAGAAAAAT
GGTAGGGCGCGGTAGTTAATTCATGCGGCTCTCTTACTCTG
TTTACATCCTAGAGCTAGAGTGCTCGGCTGCCGGCTGAGT
CTCCTCCCACTTCCCACTCCCACTCCCACTCCCACTAAAG
CGCCCTCCCGGTTCCCAAAGCAGAGGGCGTGGGGGAAAA
GAAAAAGATCCTCTCTCGCTAATCTCCGCCACCGGCCCT
TTTATAATGC
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Fig. 3. Sequence of amplified breakpoints. PCR products of tumors 2 and 7 were recovered from gel, re-amplified and subcloned for sequence analysis. In both cases, in addition to the wild-type *c-myc* gene (shown only for tumor 7, lower band), an immunoglobulin gene (IgM heavy chain switch region for tumor 2; IgG4 heavy chain switch region for tumor 7) translocated to *c-myc* was detected. The sequence of the bottom strand is shown. The adapter oligo sequence is underlined, the *c-myc* sequence is shown in regular characters with the CT-element highlighted in bold, the translocated immunoglobulin gene sequences in italic bold characters.

nism similar to that assessed here, but rejected for lack of evidence in Burkitt's lymphoma, seems to be relevant for other chromosomal translocations. In case of the t(14;18) translocation, occurring in follicular lymphoma, a non-B-DNA structure of the major breakpoint region (Bcl-2 Mbr) contains stable regions of single-strandedness. This region is vulnerable to cleavage by the RAG complex, thus proving a basis for the fragility of this translocation hot-spot [11]. So, what is the difference due to? Both, follicular as well as Burkitt's lymphoma are of germinal center origin [5]. However, in case of follicular lymphoma, the recombinase activity of the RAG1/RAG2 complex seems to be the pivotal pathogenetic principal, whereas in Burkitt's

lymphoma, the process of somatic hypermutation, occurring long after the assembly of the coding regions, is responsible for breakpoint formation [3]. Obviously, the altered chromatin structure by itself is not sufficient for the breakpoint generation.

The second aspect of this study describes a modified application of LM-PCR. The same strategy used here to map translocation breakpoints in relation to the CT-element of the *c-myc* gene can be used to map chromosomal translocations or rearrangements in any gene of interest. This assay provides several advantages over other methods used for the same purpose. Compared to Southern blotting, LM-PCR requires less DNA, allows a higher resolution, and in addition, is faster and easier to interpret. Compared to direct sequencing, LM-PCR circumvents the need of subcloning and screening genomic DNAs, and the use of an adapter primer allows amplification of a translocated gene, even when only one partner is known and when contaminated with DNA from cells with unrearranged alleles.

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