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Detection of *MYC* Gene Amplification in Malignant Lymphomas

The role of oncogene or tumor supressor gene in Epstein-Barr virus (EBV) associated malignant lymphomas (MLs) is poorly understood. We examined 36 MLs (21 EBV positive and 15 EBV negative) and 6 reactive hyperplasias for the presence of *myc* gene amplification. Polymerase chain reaction (PCR) technology was used to examine the state of amplification of the proto-oncogene *c-myc* in formalin-fixed paraffin-embedded tissues. Variable degrees of *myc* gene amplification were detected in reactive hyperplasias and MLs. However, significant increase of *c-myc* copy numbers above 3 times were only found in 12 out of 31 non-Hodgkin's MLs (38.7%), in which 6 cases were EBV positive and 6 cases were EBV negative. In conclusion, *myc* gene amplification appears to play a part in MLs but no correlation was found between EBV infection and *myc* gene amplification.

Key Words : *Gene amplification, C-myc; Lymphoma, Malignant lymphoma; Herpesvirus 4, human*

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INTRODUCTION

EBV is detected in several human cancers, such as Burkitt's lymphoma, nasopharyngeal carcinoma, gastric cancer, and peripheral T-cell lymphoma (1, 2). However, the role of EBV in the development of these cancers is still controversial because the majority of EBV infections usually occur early in life, with over 90% of the adult population demonstrating evidence of past infection (3). EBV infection is consistently found in the endemic form of Burkitt's lymphoma and the resulting activation of *c-myc* has been regarded as an essential factor for the progressive growth of the tumor cells (4). Recent study has shown that *c-myc* expression is deregulated in EBV-immortalized cells. In nasopharyngeal tumors, DNA from EBV has been consistently shown to be incorporated into the genome of tumor cells (5). Therefore, it is very interesting to research the role of oncogenes in the development of malignant lymphoma (ML) in relation to EBV infection. However, there are no systematic studies of its possible role in EBV related MLs. In this study, we investigated the incidence and degree of *c-myc* amplification with the relation between *c-myc* amplification and EBV infection in MLs.

MATERIALS AND METHODS

The study group comprised formalin-fixed paraffin-embedded lymph node biopsies of 36 MLs (31 non-Hodgkin's lymphomas and 5 Hodgkin's lymphomas) and 6 reactive hyperplasias (RHs) including 2 Castleman's disease and 1 progressive transformation of germinal centers (PTGC). All cases were selected from the surgical pathology files of Hanyang University Hospital, Seoul, Korea. Among the malignant lymphoma cases, EBER in situ hybridization positive and negative cases on screening were selected for the study. All of the cases were reviewed histologically and classified by the modified International Working Formulation (6).

Immunohistochemical studies

Immunophenotypic studies were performed on formalin-fixed, paraffin-embedded sections using the following monoclonal antibodies that are reactive in routinely processed paraffin-embedded tissues by a streptavidin- alkaline phosphatase method; a panel of monoclonal antibodies against T cells [CD43 (MT-1, BioGenex), CD45RO (UCHL-1, Dakopatts)]; B cells [CD20 (L-26, Dakopatts), MB-2 (BioGenex)]; macrophage [CD68 (Dakopatts)]; activated T, B, and Reed-Sternberg cells [CD30 (Ki-1, Dakopatts)] were used.

Detection of EBV genome in formalin-fixed paraffin-embedded tissues

In situ hybridization studies

To detect EBV nuclear RNA transcript, in situ hy-

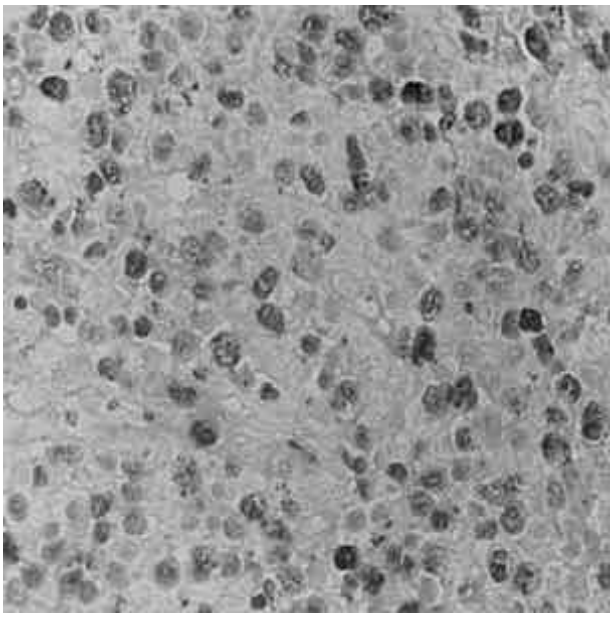

Fig. 1. EBER in situ hybridization of diffuse large cell lymphoma showing positive signals in the nuclei of neoplastic cells.

bridization studies (ISH) were performed with a fluorescein-conjugated oligonucleotide probe, EBER (Y017, Dakopatts, Denmark). Briefly, 10-μm thick sections cut from paraffin blocks of formalin-fixed tissues were mounted on glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, U.S.A). After deparaffinization and rehydration the sections were digested with proteinase K (Sigma) at a concentration of 3 μg/ml for 30 min. at 37℃, washed in water, and dehydrated in gradient ethanol. After complete drying of the slides, one drop of FITC-conjugated EBERs was applied and incubated for 2 h at 37℃. After washing twice in Tris-buffered saline (TBS) containing 0.1% Triton X-100, the slides were incubated for 30 min. at 37℃ with rabbit F (ab') anti-FITC antibody conjugated with alkaline phosphatase (1:50, Dakopatts). After washing in TBS the slides were incubated in NBT/BCIP (1:50, Dakopatts) diluted in 0.1 M TBS containing 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.0. Dark purple discoloration of the nuclei was interpreted as a positive signal (Fig. 1).

Polymerase chain reaction (PCR) amplification of EBV genome

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using a QIAamp tissue kit (Qiagen, Germany) as described in protocol. For PCR amplification of EBV genome, 20 base primers were designed to amplify 138 bp segment in the EBNA-1 region and designated EBNA1-PF: 5'-TGA TAA CCA TGG ACG AGG AC-3' and EBNA1-PR: 5'-GCA GCC AAT GCA ACT TGG AC-3'. 1 μl of genomic DNA was added to 19 μl of PreMixTM-TOP (K2016, Bioneer) PCR amplification kit. After being denatured at 95℃ for 3 min, samples were subjected to 35 cycles of amplification (30 sec at 94℃, 1 min at 58℃, 2 min at 72℃), and final extension of 15 min at 72℃. Known EBV negative and positive DNAs from lymphoma patients were used as a control.

Southern blot analysis of PCR amplified samples

PCR amplified products were visualized with UV light as a discrete single band of 138 bp fragment by staining with ethidium bromide after agarose gel electrophoresis. The PCR products were transferred to Hybond N+ membranes (Amersham, Buckinghamshire, England) by Southern blotting. Membranes underwent 5 h of prehybridization at 43℃ with a solution containing 5X SSPE (1X SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]), 0.1% sodium dodecyl sulfate). An 18 bp EBNA1 oligonucleotide probe (EBNA1P) was labelled with ^{32}P γATP using a 5'oligonucleotide end labelling kit (Amersham). Oligonucleotide probe is designed and designated EBNA1P: 5'-TCA GGA TCA GGG CCA AAA-3'. The ^{32}P-labelled probes (100 pmol) were hybridized to the blots for 12 hrs at 50℃, washed under stringent conditions and exposed to Agfa X-ray films overnight.

Detection of *myc* gene amplification in formalin-fixed paraffin-embedded tissues

PCR technology was used to examine the state of amplification of the proto-oncogene *c-myc* in formalin-fixed paraffin-embedded tissues as performed by Schreiber and Dubeau (7). Sequences from the *c-myc* gene and from a control gene, tPA, were amplified simultaneously by PCR in the same PCR mixtures.

Synthetic primers used for enzymatic amplification of *c-myc* sequences were 5'-CTC GGA AGG ACT ATC CTG CTG CCA A-3' and 5'-GGC GCT CCA AGA CGT TGT GTG TTC G-3'. The former corresponds to nucleotides 6840 to 6865 of the published *c-myc* sequence and the latter is complementary to nucleotides 6990 to 6965 of the same sequence. Primers used for enzymatic amplification of tPA sequences were 5'-TCA AAG GAG GGC TCT TCG CCG ACA T-3' and 5'-TCC TGG AAG CAG TGG GCG GCA GAG A-3'. The first extends from nucleotides 1017 to 1042 and the second is complementary to nucleotides 1167 to 1142 of the published tPA sequence. Each of the above set of primers resulted in the enzymatic amplification of sequences of a length of 147 bases. All oligomers were synthesized using an oligonucleotide synthesizer at Bioneer (Korea). Two different sets of primers, one for *c-myc*

and the other for tPA were present simultaneously in each reaction. 1 μl of genomic DNA was added to 19 μl of PreMixTM-TOP (K2016, Bioneer) PCR amplification kit. After being denatured at 95℃ for 3 min, samples were subjected to 25 cycles of amplification (1 min at 95℃, 1 min at 65℃), and final extension of 10 min at 72℃. PCR amplified products were analysed by 2% Nusieve agarose gel electrophoresis. DNAs extracted from the blood of healthy adults, HL-60 cell line, and SKOV3 cell line were used as a control.

Slot blot analysis of coamplified PCR products was performed as follows; denatured DNA samples (PCR product 10 μl, 10 N NAOH 8 μl, 0.5 M EDTA), were heated at 100℃ for 10 minutes and 1 volume of cold 2 M ammonium acetate (pH 7.0) added. The Bio-Dot SF apparatus was assembled with a Hybond N+ membrane. Equal amount of DNA samples, one for *c-myc* and the other for tPA, in each lane were transferred to Hybond N+ membrane under the gentle vacuum apply. Transferred equal amounts of DNAs to nylon membrane were hybridized with radiolabelled *c-myc* & tPA probes in the same hybridization condition. After washing at stringent conditions, autoradiography at -70℃ was performed. Quantitation of gene amplification were measured by image analyzer (Bio-Profil, France) and ratios of the two products were measured.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). The statistical significance of the difference between the group means was determined by the student's *t*-test.

RESULTS

Detection of EBV DNA using polymerase chain reaction

Histologic types and immunophenotypes of MLs are summarized in Table 1.

In this study, EBER ISH and/or EBV DNA PCR with Southern blot analysis with autoradiography positive cases were counted as EBV positive cases. EBER ISH was performed in 38/42 (33/36 MLs and 5/6 RHs) cases studied. Among them, EBER ISH positive cases were 10/33 MLs, 1/5 RHs. 4/38 EBER ISH positive cases (4/33 MLs 0/5 RHs) failed to reveal EBV DNA sequences on autoradiography.

12/38 EBER negative cases (9/33 MLs and 3/5 RHs) turned out to be EBV DNA positive with Southern blot analysis (Fig. 2). EBV bands were detected only after Southern blot hybridization in 6/42 cases (5/36 MLs and 1/6 RHs). In summary, among the 36 cases of MLs, 21 cases were EBV positive and 15 cases were EBV negative.

Table 1. Histologic types & immunophenotypes of non-Hodgkin's lymphomas

Histologic Types	Total No. of cases	Immunophenotypes	
		T	B
Follicular, small cleaved	1	0	1
Follicular, large cell	1	0	1
Diffuse, small cleaved	1	0	1
Diffuse, mixed small & large cell	12	11	1
Diffuse, large cell	9	6	3
Large cell, immunoblastic	3	1	2
lymphoblastic	1	1	0
Burkitt	3	0	3
Total(%)	31	19 (61.3)	12 (38.7)

Fig. 2. Southern blot hybridization of EBNA1 PCR products probed with γP^{32} dATP-labelled EBNA1 probe.

Results of *c-myc* and tPA PCR

Each of the above set of primers resulted in enzymatic amplification of sequences of a length of 147 base pairs in the same PCR mixtures (Fig. 3). As a control exper-

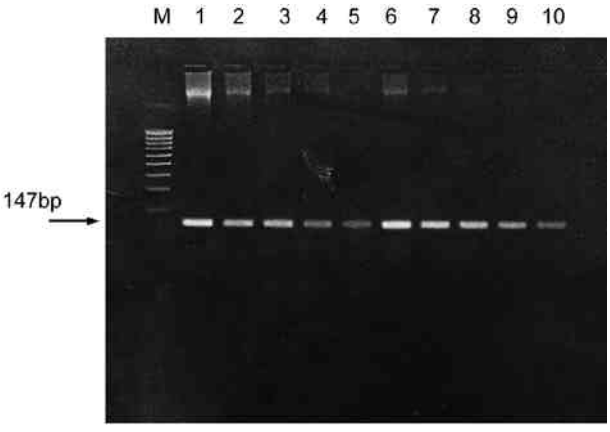

Fig. 3. DNAs were coamplified by PCR using *c-myc* and tPA primers. Each of the above set of primers resulted in enzymatic amplification of sequences of a length of 147 base pairs in the same PCR mixtures. M: DNA molecular weight marker VIII (Boehringer Mannheim)

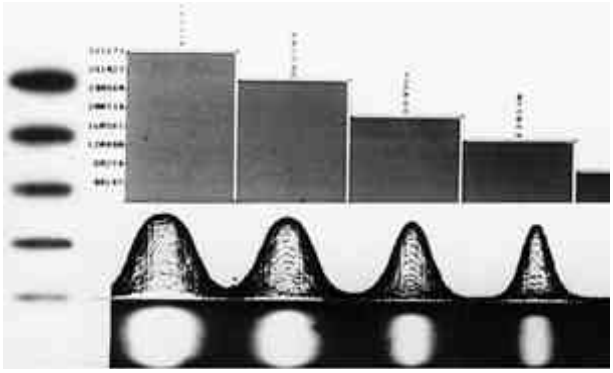

Fig. 4. Results of densitometry by image analyzer. DNAs were coamplified with serial dilutions. There were gradual decreases of gene amplification after slot blot hybridization probed with γP^{32} dATP-labelled tPA probe.

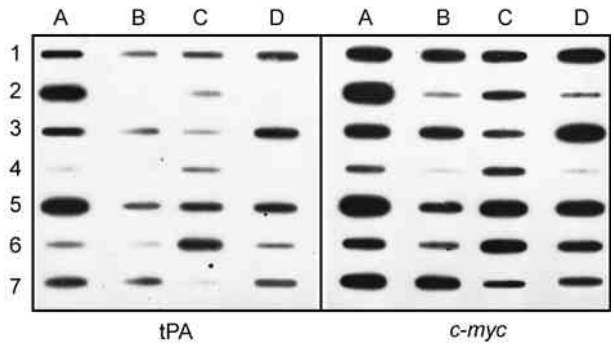

Fig. 5. Slot blot analysis of co-amplified PCR products probed with γP^{32} dATP-labelled *c-myc* and tPA oligonucleotide probes. Lane D7: negative control, DNA extracted from the blood of normal healthy adults.

iment, DNAs were coamplified with serial dilutions of DNAs and there were gradual decreases of band intensities in gel electrophoresis. This was clearly recognized after slot blot hybridization with radiolabelled *c-myc* and tPA probes (Fig. 4) and not significantly affected by the number of cycles used for the reaction. *myc*/tPA ratio was from 0.88 to 1.29 (average 1.04) in the negative control. In reactive hyperplasias, *myc*/tPA ratio was from 2.253 to 2.590 (average 2.46) except one Castleman's disease which showed 6.775 of *myc*/tPA ratio. In non-Hodgkin's MLs, *myc*/tPA ratio was from 1.334 to 9.144 (average 3.089). Among them 17 were EBV positive and 14 were EBV negative Significant increase of *c-myc* copy numbers above 3 times was only found in 12 out of 31 non-Hodgkin's MLs examined (38.7%) (Table 2) (Fig. 5). Among them 6 cases were EBV positive and 6 were EBV negative. Average *myc*/tPA ratio was 3.18 in EBV positive non-Hodgkin's MLs and 2.98 in EBV negative non-Hodgkin's MLs. In 5 Hodgkin's lymphomas, *myc*/tPA ratio was 1.550 to 2.585 (average 1.96) except one Hodgkin's lymphomas which showed 8.991. It appears that more increases of *c-myc* copy numbers were found in MLs than in RHs (Fig. 5), and in EBV positive MLs than in EBV negative MLs ($p>0.5$).

Table 2. Amplification & relative copy number of cMYC in archival specimens

Diagnosis	*c-myc*/tPA (>3.0)	Marker	EBV
1. Follicular, large, ML	6.9	B	+
2. Diffuse small cleaved, ML	4.3	B	+
3. Diffuse mixed, ML	9.1	T	+
4. Diffuse mixed, ML	3.1	T	+
5. Diffuse large, ML	3.8	B	+
6. Diffuse large, ML	3.2	B	+
7. Diffuse mixed, ML	5.3	T	−
8. Diffuse mixed, ML	4.7	T	−
9. Diffuse mixed, ML	3.5	T	−
10. Diffuse large, ML	4.2	T	−
11. Burkitt's, ML	3.9	B	−
12. Lymphoblastic, ML	3.8	T	−
13. Hodgkin's lymphomas	9.0		−
14. Castleman's disease	6.8		−

DISCUSSION

Gene amplification is a common mechanism of activation for several cellular proto-oncogenes. The additional gene copies either may be integrated into a given chromosome, in which case they appear as a homogenously staining region in conventional Giemsa-stained cytogenetic preparations, or may occur as small paired extrachromosomal bodies called double minutes (8). The main advantages of using PCR technology rather than more conventional approaches for the detection of genetic amplification are that much smaller quantities of DNA material are needed, the technique does not depend on the availability of pure intact DNA, and is readily applicable to formalin-fixed, paraffin-embedded archival specimens. However, the use of PCR technology for quantitative analyses has been limited because the quantities of PCR products obtained can be markedly altered by slight fluctuations in variables such as incubation temperatures and reagent concentrations. In the present study, two different genetic sequences were enzymatically amplified simultaneously, one corresponding to a control gene and the other to a test gene as performed by Schreiber and Dubeau (7). It has been emphasized that control genes from the same chromosome should always be used as test genes because chromosomal duplications, which are frequent in malignant tumors, otherwise would

be impossible to distinguish from true gene amplification (7-9). We found various degrees of amplification of the *c-myc* protooncogene in archival MLs and in reactive hyperplasias when tPA, which is a gene located on the same chromosome as *c-myc*, was used as a control. *Myc* is clearly important in cellular proliferation, being expressed at low or undetectable levels in resting or quiescent cells, but induced to high levels in mitotically activated cells (10). Because *c-myc* has been encountered as an activated oncogene in a wide variety of different settings, its elevated expression as a correlate of gene amplification in a number of human neoplasms provides strong support for a role of DNA amplification in the activation of cellular oncogenes in the course of tumor development (11). Amplification and overexpression of *c-myc* has been detected in HL-60 cells and in a number of other tumors including breast, stomach, and lung carcinomas, malignant neuroendocrine cells derived from a colon carcinoma, neuroblastomas, and glioblastomas (11). Amplification of *c-myc* is particularly common in breast cancers and small-cell lung carcinomas, occurring with frequencies of about 30% (11). The author previously demonstrated overexpression and amplification of *c-myc* in epidermal and melanocytic tumors (12), ovarian carcinomas (13) and in a subset of osteosarcomas (14). *Myc* deregulation in tumors may occur by different mechanisms, including rearrangement, amplification, and point mutation (10). While amplification is well known in some solid tumors, rearrangement by chromosomal translocation and point mutation are largely restricted to lymphoid neoplasms. In lymphomas with the translocation t (8;14) (q24;q32), the juxtaposition of the immunoglobulin heavy chain gene from 14q32 to the *myc* gene at 8q24 results in increased transcription of the *myc* gene because of the proximity of the immunoglobulin enhancers, and an increased mutation rate within the *myc* gene presumably owing to the hypermutational mechanisms normally associated with the immunoglobulin heavy chain gene (10). The resulting point mutations at the Pvu II restriction enzyme site in exon-1 of the *myc* gene also have an activating effect on transcription of the gene and result in loss of the recognition site for the enzyme (15, 16). Abnormal expression of *c-myc* mRNA and protein have been reported in various types of non-Hodgkin's and Hodgkin's lymphomas (17-20). There are several studies on lymphoid malignancies, indicating the degree of *c-myc* expression correlated with the proliferative index and histologic grades (21, 22). High frequency of *c-myc* gene alteration have been reported in aggressive lymphomas of lymph nodes and gastrointestinal tract (17-22). Recently, it has also been reported that amplification of *c-myc* played some role in the progression and proliferation of peripheral T-cell lymphoma (23), a case of AML (24), and follicular lymphoma (25). However, there have been few studies of the *myc* gene amplification by molecular genetic method. In this study, we investigated the incidence and degree of *myc* gene amplification in MLs. Variable degrees of *myc* gene amplification were detected in reactive hyperplasias and MLs. However, significant increases of *c-myc* copy numbers ranging from 3 to 9 times were only found in 12 out of 31 non-Hodgkin's MLs (38.7%). *Myc* gene amplification in this study occurred regardless of immunophenotypes, histologic types and histologic grades. In this study, most cases studied were intermediate to high grade lymphomas and the total number of low grade MLs studied, however, was too small to derive any conclusions regarding the true prevalence and the possible clinical significance of *myc* gene amplification. In conclusion, *myc* gene amplification appears to play a part in MLs but no correlation was found between EBV infection and *myc* gene amplification.

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