

Molecular Cytogenetic Analysis of Chemoresistant Non-Hodgkin's Lymphoma Patients with p53 Abnormalities using Fluorescence *In Situ* Hybridisation and Comparative Genomic Hybridisation

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Abstract

Background: Alterations of the p53 gene at 17p13.1 as well as the gene for a transmembrane p-glycoprotein, ABCB1 (MDR-1) at 7q21.12, have been shown to be mostly associated with the phenomenon of multi-drug resistance (MDR) in human cancers. In order to better understand the mechanisms by which chemoresistance is mediated, non-hodgkin's lymphoma (NHL) patients overexpressing p53 mutant protein and resistant to CHOP chemotherapy, NHL patients without p53 overexpression and a Burkitt's lymphoma Raji cell line with p53 overexpression have been evaluated using fluorescent *in situ* hybridization (FISH) and comparative genomic hybridization (CGH).

Methods: Three chromosomes (1, 7, and 17) known to be associated with MDR and the presence of p53 mutant protein, were analysed by FISH.

Results: No obvious chromosomal aberrations such as translocations were found in any of the patients when compared to healthy individuals, which suggests that the three selected chromosomes might not be specifically related to NHL, with or without p53 overexpression. For CGH, gains and losses of chromosomal material have been identified and the changes were not only limited to the three selected chromosomes associated with MDR. A detailed analysis of the recurrent aberrations shows that most of the NHL patients have alterations on the chromosome arms 1p, 6q, 7q, 20q, 22q, and Xp, whereas patients with p53 overexpression predominantly show aberrations on 4p and 17q.

Conclusion: Further characterisation of the genetic regions identified might more closely contribute to our understanding of acquired MDR in NHL. Alterations in the three evaluated chromosomes may be prevalent in other tumours. In the present study, using FISH and CGH, there was insufficient difference between NHL patients with and without p53 overexpression.

Keywords: cellular biomarker, chemoresistance, P-gp, p53

Introduction

One of the goals for finding better chemotherapy treatments is to understand how tumor cells can become chemoresistant. The analysis of how human cancers evade chemotherapy has revealed a variety of cell-based genetic changes resulting in chemoresistance. Despite enormous research efforts, resistance to chemotherapeutic agents, inherent as well as acquired, continues to pose major obstacles toward the successful chemotherapeutic treatment of various human cancers.^{1,2} Chemoresistance is a major difficulty during the treatment of many human cancers.² This phenomenon occurs in numerous types of non-hodgkin's lymphoma (NHL).³ Several multi-drug resistance (MDR) mechanisms have been identified such as increased drug efflux, alterations in nuclear targets such as p53, modifications of

DNA repair systems, apoptotic regulatory systems, gene amplification, enhanced intracellular drug detoxification and drug target regulation systems.⁴⁻⁶

NHL is a heterogeneous group of tumors of the lymphatic system.^{7,8} The standard regimen for the treatment of NHL patients is CHOP,⁹ which comprises cyclophosphamide, hydroxy daunorubicin (doxorubicin), Oncovin[®] (vincristine), and prednisolone.

Cytogenetic analysis of NHL utilizing G-banding and fluorescent *in situ* hybridization (FISH) has identified recurring clonal chromosomal abnormalities that are of biological and clinical significance.¹⁰ Cytogenetic analysis has also identified several drug resistance genes including ABCB1. The human ABCB1 (MDR-1) gene is located on the long arm of chromosome 7 in the region of 7q21.12 and encodes for a 170-kDa membrane-associated protein (ATP-binding cassette transporter, subfamily B, member 1).^{11,12} Overexpression of PGP the product of the ABCB1 gene, has been linked to the failure of chemotherapy in many human cancers including lymphomas.¹³ There is also strong evidence that the p53 gene is linked to drug resistance (14); p53 is located on the short arm of chromosome 17, in the region of 17p13.^{15,16} In addition, aberrations of chromosomal bands 1p36 and 1q11-q23 are among the most common chromosomal alterations in NHL.¹⁷ Therefore the present study, using a FISH approach, first targeted these three chromosomes (1, 7, and 17), which are mostly associated with the MDR phenomenon in NHL. Subsequently, using comparative genomic hybridization (CGH) has been employed to screen for ab-

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normalities linked to MDR in the entire genome by comparing isolated genomic DNA from tumour cells to control DNA.¹⁸⁻²⁰ Some studies have already successfully used FISH and CGH techniques in parallel to the standard cytogenetic methods for the characterisation of genetic aberrations associated with the acquisition of drug resistance in drug-resistant cell lines.^{21,22} Thus the identification of such genetic markers in NHL patients may further strengthen the identification of the chromosomal regions, which may harbor putative genes responsible for MDR²³ as valuable biomarkers. This may increase the efficacy of chemotherapy and predict the best treatment for relapsed patients with chemoresistance, particularly for NHL patients.

The present study examines NHL patients with and without p53 overexpression, referred to as p53⁺ and p53⁻,²⁴ with FISH and CGH analysis to determine any difference in chromosomal regions that might be used as biomarkers for acquired drug resistance in NHL patients.

Material and Methods

Biological samples

The Haematology Department at Airedale Hospital, Steeten, UK provided samples from NHL patients as well as control individuals and performed the pre-screening immuno-cytochemistry tests²⁵ by using the primary antibody DO-7 specific for the p53 mutant protein (EnVision™ DAKO K1393, Dako, UK). By definition, p53⁺ patients showed greater than 10% and p53⁻ patients as well as control individuals showed less than 10% of the cells staining for p53 mutant protein. All patients had previously received several courses of CHOP chemotherapy but no additional treatment such as radiotherapy. Only p53⁺ patients showed resistance to the CHOP regimen. For our study, lymphocytes from normal healthy donors served as a negative control while the Burkitt's lymphoma Raji cell line served as a p53⁺ model for NHL since it overexpresses p53 protein.²⁶

Human blood

Human peripheral blood samples were obtained with informed consent and prior ethical approval. For FISH studies, blood was obtained by venepuncture from healthy volunteers and sixteen NHL patients (eight p53⁺ and eight p53⁻ patients). Furthermore, four NHL patients, (two p53⁺ and two p53⁻ patients) and a Raji cell line were used for CGH studies.

Lymphocyte isolation: chromosome preparation and genomic DNA extraction

Peripheral blood samples (3 mL) were obtained with informed consent and prior ethical approval from LREC at the Airedale Hospital. To standardise conditions for working with clinical samples the collected blood was kept at room temperature in heparinized tubes for about 20 hr. Isolation of lymphocytes was carried out using Lymphoprep (Axis Shield, Norway). Separated lymphocytes were washed and subsequently resuspended either in 1 mL RPMI 1640 for culturing with a subsequent metaphase preparation (FISH studies) or in 200 µL PBS for DNA isolation (CGH studies). For the 96 hr cultures (37°C, 5% CO₂), 0.5 mL lymphocyte suspension was added to 4.5 mL culture medium containing RPMI 1640, 10% foetal bovine serum, 26 µg/mL phytohemagglutinin, 1% penicillin/streptomycin mixture (all from Gibco, UK), 10 µM 5-bromo-2'-deoxyuridine (Sigma, UK) and 2 U/mL IL-2 (Roche, UK).²⁷

For the last three hours of the incubation, the medium was supplemented with 0.4 µM colcemid (Roche, UK). Processing of the culture and the succeeding metaphase preparation on glass slides was performed according to standard procedure.²⁸ For genomic DNA isolation, DNA was extracted from isolated lymphocytes from each clinical sample. For the Raji cell line, 1–2×10⁶ cells were used for isolation. All DNA was extracted with a DNA isolation kit (Qiagen, UK) according to the manufacturer's instructions.

FISH, DOP-PCR and CGH

FISH analysis was performed with slight modifications as described by Cambio Ltd. (Cambridge, UK) for whole chromosome painting. Direct-labelled whole chromosome painting probes for human chromosomes 1, 7, and 17 were obtained from Cambio and used in all FISH experiments. At least 50 metaphases from each clinical sample and the cell line were analysed for chromosomal aberrations involving the three chromosomes. Briefly, the following steps were employed for FISH²⁹: pepsination of the metaphase spreads, denaturation of the target DNA (metaphase spreads) and DNA probes, hybridisation over two days, counterstaining as well as image analysis with a fluorescence microscope.

Often only small amounts of tumor DNA from cells were available, therefore it was advantageous to amplify the tumor DNA by DOP-PCR.³⁰ In brief, DOP-PCR was employed to increase the amount of DNA and to incorporate nucleotides conjugated to fluorochromes into the probe DNA. Four cycles were carried out in a 10 µL reaction mixture using the UNI primer and Thermo Sequenase DNA polymerase (Amersham, USA), followed by 25 cycles in a 50 µL reaction volume using UNI primer and AmpliTaq polymerase. CGH was performed with minor modifications according to a previously described method.³¹ Briefly, tumor DNA and normal DNA was labelled by nick translation with Biotin and Digoxigenin, respectively. Equal amounts (500 ng) of labelled tumor and normal DNA were denatured together with 15 µg cot-1 DNA (Gibco, USA), dissolved in hybridisation mix and co-hybridized for 72 h at 37°C to denatured metaphase chromosomes on commercially available slides (Vysis, UK). Metaphases were counterstained with DAPI (4,6-diamidino-2-phenylindole, Sigma, Germany).

Digital image analysis of the CGH

A Zeiss fluorescence microscope equipped with a multicolor quantitative image processing system (ISIS, MetaSystems, Alt-lussheim, Germany) was used for the acquisition and for evaluation of the hybridized metaphases. Black-and-white images from the green, red, and blue fluorescence channels were acquired from a minimum of ten metaphases of the counterstained chromosomes and the fluorochromes (tumour DNA labelled with FITC, reference DNA with Cy3 and chromosomes counterstained with DAPI). The chromosomes were karyotyped on the basis of the inverted black-and-white DAPI image. Fluorescence intensity profiles of green and red fluorescence were calculated by integrating fluorescence values across the chromosome width along the medial axis and the green-to-red ratio of each chromosome was plotted as a function of distance from the p-arm telomeric region to the q-arm telomeric region. Gain of chromosomal regions was assumed at ratio of >1.15 or loss was defined at chromosomal regions where the hybridisations resulted in a ratio of <0.85. Heterochromatic regions and the entire chromosome Y were excluded from analysis.

Table 1. CGH analysis in four NHL patients (two p53⁺ and two p53⁻ patients) and the Raji cell line.

Patient/NHL Case no.	Age	Sex	p53 status	Diagnosis	Amplification	Deletion
p53 ⁺ I	59	Female	+	Systematic marginal zone lymphoma	4p13 4q13 Xq28	17q25 20q12-q13.2
p53 ⁺ II	66	Male	+	Diffuse large B cell lymphoma	1p22-p31.1 1p32-p35 1q21-q23 1q32 4p14-p15.3 4q27-q28 6q12-q14 6q22-q25 7q11.2-q32 12q24.1-qter 14q11.2-q31 15q14-q24 17q 19p13.1 19q13.3-qter 20q 22q11.2-q12	Xp21.2-p22.2
p53 ⁻ I	69	Male	-	Diffuse large B cell lymphoma	6q14-q16 7q11.2-q21	
p53 ⁻ II	69	Male	-	Marginal zone lymphoma	1p36.1-pter 3p21 3p24-p25 6p 6q14-q15 6q22-qter 10q22 10q24-qter 11q13-qter 12q13-q14 16p12-p13.2 16q22-qter 20q12-q13.1 21q21-qter 22q11.2-12	1p13-p22 4q22-q28 5q11.2-q23 7q11.2-q21 Xp21.1 Xq
Cell line	Derived from 17 year old African boy	Male	+	Burkitt's lymphoma cell line	3q23-qter 6q 7p 7q11.2-q22 20	8q24.1-qter

Results

FISH analysis

Fifty metaphases from each patient and at least 100 metaphases from control donors were evaluated. No obvious aberrations such as gaps, breaks, interchromosome bridges, dicentrics, acentric fragments or deletions were found in the NHL patients as well as in the control group (data not shown).

CGH analysis

For CGH, the following results for the clinical patients p53⁺ I and II, and p53⁻ I and II as well as for the cell line are shown in Table 1. Gains and/or losses were identified, but the changes were not only seen within the three chromosomes 1, 7, and 17, but also in others. The genetic status of all gains and losses in the clinical samples and the NHL p53⁺ cell line model (Raji) were examined to determine if there were differences in resistant and non-resistant phenotypes.

For the p53⁺ patients, one patient, p53⁺ I, had few losses (17q25

and 20q12-13.2) and gains (4q13, 4q13 and Xq28) of chromosomal material. For patient p53⁺ II, however, there were multiple gains found within the genome. These gains included large areas on chromosome 1p (1p22-p31.1 and 1p32-p35) as well as on the q-arm (1q21-q23 and 1q32). Minor gains were seen on chromosome 4 within bands 4p14-p15.3 and 4q27-q28. For the C-group chromosomes, gains were found within the chromosome bands 6q12-q14, 6q22-q25, 7q11.2-q32, and 12q24.1-qter. Almost the whole q-arm of chromosome 14 and 15 (14q11.2-q31, 15q14-q24) showed gains in chromosomal material in the p53⁺ II patient. For chromosome 17 and 20 the complete q-arm was amplified. Smaller gains were also seen in the chromosomal bands 19p13.1, 19q13.3-qter and 22q11.2-q22. The only loss of X-chromosomal material was observed in the bands Xq21.2-q22.2.

For the p53⁻ patients, patient I also showed lower amounts of gains and losses (6q14-q16 and 7q11.2-q21). In patient p53⁻ II more gains than losses of chromosomal material were seen. For the larger chromosomes minor gains were seen within bands

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1p36.1-pter, 3q21, and 3p24-p25. However, larger sections of chromosomal material within this patient were lost: 1p13-p22, 4q22-q28, and 5q11.2-q23. Within the C-group chromosomes, chromosome 6 showed the highest amount of gains (on the complete 6p arm, 6q14-q15 and 6q22-qter). Smaller gains were seen at 10q22, 10q24-qter, 11q13-qter, and 12q13-q14 as well as a loss in the bands 7q11.2-q21. For the rest of the chromosomes only minor gains were observed (16p12-p13.2, 16q22-qter, 20q12-q13.1, 21q21-qter, and 22q11.2-q12). On chromosome X, large areas appeared to be deleted in the leukaemia patient, Xq21.1 as well as the complete p-arm.

For the Raji cell line, larger gains on a few chromosomes were observed within the chromosomal areas 3q23-qter, 7q11.2-q22, the complete chromosomal arms of 6q and 7p as well as the whole chromosome 20. A small loss of chromosomal material was seen on chromosome 8 at 8q24.1-qter.

Discussion

Tumor development proceeds via clonal divergence with selection for cells with a proliferative advantage, metastatic potential or a drug resistant phenotype. FISH as well as CGH allows the visualisation of the genetic make up of individual cells within their histological context. Therefore, these molecular techniques have the potential to study a number of genes such as *p53* and *ABCB1* that have been implicated in drug resistance.³² Identification of these genes may lead to a clinical model for predicting the acquisition of drug resistance in human cancers such as NHL.

Chromosomes only become visible and suited for the classical chromosome analyses during the metaphase stage of the cell cycle; therefore, to culture lymphocytes from peripheral blood is the most common method for simultaneously obtaining many cells in the metaphase stage. Normally, lymphocytes are mitotically inactive in G₀ phase of the cell cycle; hence, they have to be externally stimulated by mitogen phytohaemagglutinin to divide. However, for preparation of chromosomes from NHL patients' blood it was impossible to succeed without supplementing the culture medium with interleukin-2 to initiate proliferation of the T-lymphocytes *in vitro*.²⁷ This approach showed benefits in the cytogenetic examination of the NHL patients' cells in this study. With FISH using whole chromosome painting probes, no differences between p53⁺ and p53⁻ patients versus a healthy control individual were detected within the targeted chromosomes 1, 7, and 17. However, we cannot exclude cryptic translocations,³³ which are not detected because they are below the sensitivity threshold of about 10 Mbp for whole-chromosomal paint FISH. Conclusively, there was no significant difference in the incidence of *p53* deletions and/or translocations in p53⁺ and p53⁻ patients, which has been shown previously.⁵ This might be because the three targeted chromosomes associated with MDR and the presence of *p53* mutant protein are not specifically altered on a large scale within the assessed NHL patients, neither with nor without *p53* protein overexpression. Reports can be found showing no differences³⁴; however, deletions within the region 17p13 have been reported.³⁵

In our study, CGH evaluation was able to detect imbalances along many chromosomes of the NHL patients. We found that amplifications (gains) were more frequent than deletions (losses) within the NHL patients' lymphocyte DNA. However, gains and losses were not confined to chromosomes 1, 7, and 17 but there were also other chromosomes affected. As shown in Table 1, some

chromosomal aberrations can be recurrently found either being common for all the NHL patients or only for the p53⁺ patients who overexpress p53. For this study, it seems that parts of the chromosome arms 1p, 6q, 7q, 20q, 22q, and Xp were predominantly affected by NHL. Rearrangements at the 1p36 locus, which are very common in NHL, have been previously reported in NHL patients^{36,37} and lymphoblastoid cell lines.³⁸ Certain genes at this locus seem to strongly contribute to the pathogenesis of NHL: the *PRDM16* (*MEL1*, 1p36.32) gene encoding for a Zn-finger transcription factor, the p53-related tumor protein *TP73* (1p36.32) gene³⁷ as well as the caspase gene *CASP9* (1p36.21). It appears that the genetic variation in caspase genes generally play a significant role in the aetiology of NHL.³⁹ There seem to be also two important loci on the q-arm of chromosome 1 where imbalances can be detected in NHL patients, namely within the bands 1q21-q22 and 1q31-q32.^{40,37,41} Within these regions oncogenes like *BCL9* (1q21.1), *MUC1* (1q22), and *MDM4* (1q32.1) can be found. *MDM4* together with *MDM2* plays an important role in apoptosis via p53 binding and the overexpression of this specific inhibitor leads often to inactivation of p53.⁴² We found amplifications within these important regions on chromosome 1: patient p53⁺ II showed gains at 1p22-p35, 1q21-q23 as well as 1q32 and patient p53⁻ II at 1p36.1-pter and a loss of chromosomal material at 1p13-p22. However, some of the abnormalities found (i.e., amplifications at 1p22-p35 and a deletion at 1p13-p22) were outside of these common chromosome 1 aberrations in NHL, but appear to be nonetheless relevant in terms of chemoresistance as the genes for two trans-membrane transporter proteins, *ABCD3* and *ABCA4*, are located within this region. Also, chromosome 6q aberrations seem to contribute to the general NHL phenotype. We found gains within the regions 6q12-q14 and 6q22-q25 in patient p53⁺ II, 6q14-q16 in patients p53⁻ I and II and 6q22-qter for patient p53⁻ II as well as the entire 6p arm. The Raji lymphoma cell line that served as a p53⁺ control also showed an amplification of the complete q-arm of chromosome 6. The mainly affected regions were at the q-arm around 6q14 and distal to 6q22. These regions contain many genes of the mitogen-activated kinase pathways like *MAP3K7* (6q15) controlling apoptosis, *MAP3K5* (*ASK1*, 6q23.3) and *MAP3K4* (6q26) as well as the tumor protein D52 gene *TPD52L1* (6q22), which can interact with *MAP3K5*.⁴³⁻⁴⁵ Also, the gene *CASP8AP2* is located at 6q15, the product of which can interact with caspase 8 in the apoptotic pathway.⁴⁶ Changes on 6p have been reported in large B cell lymphoma⁴⁷ as well as deletions at 6q26-q27 for B-cell NHL.⁴⁸ Genomic aberrations such as gain/amplification within the region 7q11.2-q21 will also be acquired upon tumor transformation.⁴⁹ In the present study, a common and important hotspot for an imbalance on chromosome 7 seems to fall exactly into this region. Patient p53⁺ II and patient p53⁻ I showed gains at 7q11.2-q32 and 7q11.2-q21, respectively, whereas for patient p53⁻ II this region on chromosome 7 (7q11.2-q21) was deleted. The Raji cell line on the other hand also showed a 7q11.2-q22 and complete 7p amplification. Interestingly, the region 7q11-q21 contains various oncogenes as well as genes, which seem to play a crucial role in MDR: two genes at the locus 7q21.12, *ABCB1* (*MDR1*) and *ABCB4* (*MDR3*), code for ATP-binding cassette (ABC) transporter proteins associated with MDR.⁵⁰ Additionally, genes such as the oncogene-like *BCL7B*,⁵¹ the metalloprotease-coding *STEAP2*⁵² and cell-cycle regulating *CDK6*⁵³ are also located within this region on chromosome 7 and contribute to cancer progression and most likely to the NHL phenotype. In the Raji cell line, a trisomy 20 was found as

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well as gains on 20q. The complete 20q-arm for patient p53⁺ II and the region 20q12-13.1 for patient p53⁻ II have also been amplified. On the other hand, patient p53⁺ I showed a deletion in the same region 20q12-13.2. Particularly, imbalances on the q-arm of chromosome 20 are apparent in NHL.⁵⁴ Additionally, gains within the region 21q21-qter of patient p53⁻ II as well as within 22q11.2-q12 in both p53⁻ II and p53⁺ II have been found. It appears that a variety of events is important for forming a cancer phenotype like NHL that involves certain oncogenes and cell-cycle genes.⁵⁵

In addition to these aberrations other chromosomes have also been affected, however, imbalances on 4p and 17q seem to play an apparent role for the p53 overexpression, at least in NHL patients. Besides two affected loci on the p-arm of chromosome 4 (4p13-p15), a common denominator in terms of genetic imbalances for patients with p53 overexpression seems to be alterations within the q-arm of chromosome 17. For chromosome 17, gains have been previously found with conventional methods⁵⁶ and by using CGH.⁵⁷ In our study, patient p53⁺ I showed a deletion at 17q25 while patient p53⁺ II showed a gain of the complete q-arm of chromosome 17. Some NHL phenotypes seem to show a duplication of the q-arm in the form of an isochromosome 17q.⁵⁸ Different breakpoint cluster regions have been identified on chromosome 17 being located close to or within the centromere. Low copy repeat numbers in certain regions may be one of the factors for the increased instability that may trigger the formation of an i(17q) in neoplasms.⁵⁹

Understanding the genetic events in cancer cells that underlie sensitivity and resistance to treatment with chemotherapeutic agents is important from a biological as well as a clinical point of view. CGH has typically been applied to identify chromosomal aberrations in DNA of NHL samples relative to normal genomic DNA.⁶⁰ CGH is also a valuable and accurate method for the detection and localization of chromosomal abnormalities associated with different kinds of acquired MDR as shown in the present study and by several other authors.

Despite the fact that whole-chromosome paint FISH analysis did not detect alterations within chromosomes 1, 7, and 17, CGH analysis revealed imbalances within these chromosomes. Alterations within chromosomes 1 and 7 seem to be closely associated with the NHL phenotype, whereas alterations within chromosome 17 might give rise to the p53⁺ phenotype. This suggests that changes within these three chromosomes may well contribute towards the phenotype of our NHL, but not exclusively. We found amplifications and deletions in all three specified chromosomes by CGH as well as in other chromosomes, which involve important genes. No large rearrangements but rather gains and losses within certain loci carrying for instance important transporter genes are most likely the reason for chemoresistance in NHL. Our CGH results also suggest recurring aberrations within certain loci across the genome. It particularly seems that imbalances on chromosome arms 1p, 6q, 7q, 20q, 22q and most likely Xp add to the phenotype of NHL in general whereas aberrations within 4p and 17p can be predominantly found in p53 overexpressed (p53⁺) NHL patients. Other chromosomes, which have also been affected in this study, may also contribute to the NHL phenotype and/or chemoresistance; however, the limited number of available patients allowed no explicit evaluation. FISH and CGH are modern techniques, which may help in future studies in finding important biomarkers in terms of chemoresistance. They may provide a tool for clinics to improve choice of chemotherapy for cancer patients, in general.

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