DHFR and MSH3 co-amplification in childhood acute lymphoblastic leukaemia, in vitro and in vivo

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The MSH3 and dihydrofolate reductase (DHFR) genes, located on chromosome 5, share a common promoter but are divergently transcribed. Dysregulation of the mismatch repair (MMR) pathway has been found to occur in cell line models due to co-amplification of MSH3 as a coincident effect of DHFR amplification, acquired as a mechanism generating resistance to methotrexate (MTX). The increased levels of MSH3 perturbed MutS\(\alpha\) function resulting in hypermutability and increased resistance to thiopurines, drugs whose cytotoxic effects are triggered by MutS\(\alpha\). The relevance of this phenomenon in clinical samples is unknown but is extremely pertinent in childhood acute lymphoblastic leukaemia (ALL) in which children are exposed for prolonged periods to both MTX and thiopurines such that a single amplification event involving both the DHFR and the MSH3 genes may cause chemotherapeutic resistance to both agents. Thus, we have generated a leukemic cell line (PreB697) and a normal human lymphoblastoid cell line (TK6) that are resistant to a pharmacologically relevant dose of MTX and show that while increased DHFR levels result in MTX resistance, the associated increased levels of MSH3 are insufficient to perturb MutS\(\alpha\) functionality, in terms of MMR capacity or 6-thioguanine sensitivity. In addition, we show that although low-level DHFR amplification occurs alone in a significant number of samples, both at disease onset and relapse, co-amplification of both MSH3 and DHFR is rarely found in primary ALL samples, even after prolonged MTX therapy and is not at a sufficiently high level to perturb MMR function.

Introduction

The antifolate, methotrexate (MTX) has been a mainstay of treatment in childhood acute lymphoblastic leukaemia (ALL) for >40 years. It acts as a tight binding inhibitor of the enzyme dihydrofolate reductase (DHFR) that catalyses the reduction of dihydrofolate to tetrahydrofolate in the biosynthesis of thymidylate and purine precursors (1). Cell line models implicate DHFR amplification as the predominant mechanism of resistance to MTX and two studies have suggested that increased DHFR levels may also occur in clinical samples. In one of these, increased expression was associated with low-level DHFR amplification (2,3).

Interestingly, the DHFR gene is situated next to that for the MSH3 (mutS homologue 3) at 5q14.1. They share a common promoter but are divergently transcribed (4). MSH3 is a member of a family of highly conserved genes that are crucial to postreplication repair of DNA mismatches (5,6). In eukaryotic cells, repair is initiated by binding of a heterodimer formed between MSH2 and MSH6 (the MutS\(\alpha\) complex) to a mispaired base. A second heterodimer complex (MutS\(\beta\)) is then recruited, in addition to the detection of base–base mispairs, the mismatch repair (MMR) system is able to detect small (+1 bp) insertion/deletion loops (IDLs) that arise due to polymerase slippage during replication (7). Larger IDLs (1–10 bp) are recognized by the MutS\(\beta\) complex which comprises a heterodimer formed between MSH2 and MSH3. As with base–base repair, the process is completed with the recruitment of the MutL\(\alpha\) complex.

Recently, studies of cell line models with DHFR amplification have revealed a concomitant co-amplification of MSH3 with a resultant increase in MSH3 protein levels. This phenomenon resulted in sequestration of MSH2 into the MutS\(\beta\) complex because MSH6 and MSH3 compete for MSH2, leading to the degradation of unbound MSH6 and a significant alteration in the relative intracellular levels of MutS\(\alpha\) and MutS\(\beta\), thereby lowering base–base repair capability while preserving IDL repair (8–10). Loss of base–base repair due to DHFR/MSH3 amplification resulted in hypermutability, with a 100-fold increase in mutation rate. In addition, there was chemoresistance to 6-thioguanine (6-TG), a thiopurine that results in the incorporation of fraudulent nucleotides into newly synthesized DNA and whose cytotoxic effect is mediated, at least in part, through recognition by the MutS\(\alpha\) complex (11–14).

These observations could have major implications for children undergoing chemotherapy for ALL because the treatment regimen involves prolonged exposure during maintenance phases to both MTX and 6-mercaptopurine, a thiopurine with a similar mode of action to 6-TG. Thus, a single genetic event that results in the co-amplification of the DHFR/MSH3 in leukemic blasts may cause co-resistance to both the key chemotherapeutic agents.

To investigate this, we have generated two sublines from the leukemic cell line (PreB697) and a normal human lymphoblastoid cell line (TK6) that are resistant to a pharmacologically relevant dose of MTX (15) and have characterized their MMR capability and thiopurine sensitivity. In addition, we have compared DHFR and MSH3 gene copy number in these lines with a cohort of clinical samples at diagnosis and at relapse using a sensitive, quantitative, real-time PCR method.

Materials and methods

Cell lines

Cell lines were obtained from ECACC, ATCC or in the case of PreB697 a generous gift from Prof. R Kofler (Innsbruck, Austria) and were cultured in RPMI 1640 supplemented with 1-glutamine and 10% fetal bovine serum (FBS). Details of the cell lines used are given in Supplementary Table I. For generation of MTX-resistant sublines of TK6 and PreB697, medium was supplemented with increasing concentrations of MTX over a period of 5 months, from 5 nM rising to 40 nM. MTX was then withdrawn from the sublines, designated TK6/MTX and PreB697/MTX, and cell samples were taken three times over a period of 2 months for repeat gene copy number analysis. All cell lines were regularly tested for mycoplasma contamination using the MycoAlert kit (Cambrex, Wokingham, UK).

Patient samples

Bone marrow samples from patients at presentation and relapse were processed within 2–3 h of collection. All relapse samples were from patients who had been treated with MTX. Lymphoblasts were separated by density gradient centrifugation over Lymphoprep (Nycomed, Birmingham, UK). Separated cells were washed in phosphate-buffered saline, counted and percentage of blasts determined by cytological examination after May–Grunwald–Giemsa staining. All samples had >90% blasts. Patient samples were obtained according to local ethical guidelines.

Real-time Q-PCR for determination of gene amplification

Cells were washed 2× in phosphate-buffered saline and DNA was extracted from pellets of 1 × 10\(^6\) cells using the QIAamp DNA mini kit (Qiagen, Crawley, UK) and quantified using a NanoDrop\textsuperscript® ND-1000 spectrophotometer

Abbreviations: ALL, acute lymphoblastic leukaemia; DHFR, dihydrofolate reductase; FBS, fetal bovine serum; IDL, insertion/deletion loop; MMR, mismatch repair; mRNA, messenger RNA; MTX, methotrexate; 6-TG, 6-thioguanine; TBP, TATA box binding protein.

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(LabTech, Ringmer, UK). Real-time quantitative PCR was performed using the GeneAmp® 5700 Sequence Detection System (Applied Biosystems) according to the manufacturer’s recommendations. Primers and probes were designed using Primer Express software and were synthesized by Applied Biosystems (Supplementary Table II). The rationale behind the method used is detailed at length in a paper by Bièche et al. (16). We applied this procedure to determine the copy number of our target genes, DHFR and MSH3 in addition to two control genes, ATP10A and SH3GL3, selected on the basis that they were located on chromosome 15, a region in which genetic alterations in childhood ALL rarely occur (17) (S.Sulong, unpublished observations). The gene ATP10A was used as an internal reference gene and SH3GL3 as a control target as no amplification of this gene should be detected. This ratio of the copy number of the target gene to the copy number of the ATP10A gene normalizes the amount and quality of the genomic DNA. Therefore, the level of amplification (N) was determined as follows:

\[
N = \frac{\text{Copy number of target gene}}{\text{Copy number of reference gene (ATP10A)}}
\]

A standard curve was constructed using PCR products from the target and reference genes. These were quantified spectrophotometrically, pooled and serially diluted 10-fold in mouse genomic DNA (Roche, Welwyn garden city, UK) at a constant concentration of 2 ng/µl (16). Each dilution point was aliquoted and stored at −80°C such that a single aliquot could be used for each assay thereby avoiding freeze/thaw cycles. The standard curve was validated by analysing a 20 ng calibrator of human genomic DNA (BD Biosciences) in each assay. Reactions (25 µl) were set up for each sample in triplicate using 1× TaqMan Universal master mix and 1 µl test DNA diluted to 20 ng/µl. For each primer and probe set, optimization was carried out using a protocol supplied by Applied Biosystems. Each assay included a five-point standard curve, a non-template control, 20 ng calibrator DNA and 20 test samples. The relative amplification was determined from the standard curve using the above formula.

**Real-time RT–PCR to determine MSH3 messenger RNA levels**

RNA was extracted from washed cells using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions and quantified spectrophotometrically. Two hundred and fifty nanograms was reverse transcribed using the TaqMan reverse transcription kit (Applied Biosystems) and random hexamers, according to the manufacturer’s instructions. cDNA was stored at −20°C until required.

TATA box binding protein (TBP) was selected as the endogenous RNA control for the quantification of MSH3 expression, based on the fact that there are no known TBP pseudogenes and that the transcript levels are similar. TBP primers and probes were available from Applied Biosystems and those for MSH3 were designed using Primer Express software and synthesized by Applied Biosystems. Primer sequences are given in Supplementary Table II. A standard curve was constructed by preparing cDNA from PreB697/MTX cells. cDNA prepared from 20 ng/µl RNA was diluted to 10, 5, 2.5, 1 and 0.4 ng/µl equivalents in Escherichia coli tRNA to ensure that the total RNA was the same in each standard. Aliquots were stored at −20°C.

PCR reactions (25 µl) were set up in duplicate for each data point. Each run included the five standard curve points, one non-template control, one calibrator cDNA and 17 test samples. Using this format, MSH3 and TBP could be analysed on the same 96-well plate. All PCR reactions were performed on the same instrument as the quantitative-PCR assays using standard cycling conditions. For each experimental sample, the amount of target and endogenous reference was determined from the standard curve. The relative gene target expression level was then normalized to a normal human cDNA calibrator, prepared from RNA extracted from TK6 cells. Each of the normalized target values was then divided by the normalized calibrator value to give the final relative expression levels:

\[
\text{Re}1\text{ MSH3} = \frac{\text{MSH3 sample}}{\text{MSH3 calibrator}}\times \frac{\text{TBP sample}}{\text{TBP calibrator}}
\]

**Western blotting**

Western blotting was carried out to verify the expression of DHFR and MSH3 protein in parental and MTX-resistant cell lines using standard techniques. Antibodies used were all mouse monoclonals and included anti-DHFR (BD Biosciences) and anti-actin (Calbiochem, Nottingham, UK). The secondary antibody was a horseradish peroxidase-conjugated antirabbit (BD Biosciences). Development was carried out using enhanced chemiluminescence (Amersham, Chalfont St Giles, UK) according to the manufacturer’s instructions.

**In vitro MMR assay**

DNA MMR was assessed as described previously (18,19). Briefly, 5 ng of an M13mp2 phage heteroduplex substrate containing either a G-T mispair or Δ5 bases was incubated with 50 µg of cell lysate. The repaired or unrepairpd substrate was purified and electrophoresed into MMR-deficient E.coli. Transformed bacteria were then plated out with the z-complementation E.coli strain CH50 onto minimal agar plates supplemented with isopropyl β-D-thiogalactopyranoside and X-Gal. The resulting plaques were scored as pure blue, mixed blue or clear. Reduction in the percentage of mixed plaques and increase in pure blue or clear plaques is indicative of MMR competence. Repair efficiency was calculated as 1 − (% mixed plaques in test sample divided by % mixed plaques in a water-only control). The M13mp2 phage derivatives and E.coli strains were kindly provided by Dr Thomas Kunkel (National Institute of Environmental Health Sciences, Research Triangle Park, NC).

**Sensitivity to 6-TG and MTX**

Ninety-six-well plates were seeded with 2 × 10⁴ cells per well/200 µl medium (RPME + 10% fetal bovine serum) with varying concentrations of 6-TG (5 µM to 39 nM) or MTX (10 µM to 0.1 nM). Each assay point was set up in triplicate. Control wells were set up with the control vehicle, DMSO in the case of 6-TG and 0.1% NaOH in the case of MTX. Cells were incubated for three culture doublings, then 40 µl CellTiter 96® Aqueous One solution (Promega, Southampton, UK) was added to each well. The absorbance at 490 nm was measured after 2 h incubation using a Spectramax 250 plate reader (Molecular Devices, Wokingham, UK). The percentage survival was calculated from the absorbance measurements at 490 nm, using the control vehicle as 100%. Survival curves were constructed from the mean of three separate assays using GraphPad Prism software and the EC50 values calculated.

**Results**

**Determination of DHFR and MSH3 copy number and protein levels in MTX-resistant cells**

Protein levels of MSH3 and DHFR were compared in parental and MTX-resistant lines by western blotting, using actin as a loading control (Figure 1A). For MSH3, both PreB697 and TK6 showed
high levels in the parental lines with an obvious increase in both MTX-resistant sublines. For DHFR, protein levels in the PreB697/MTX-variant line were also higher compared with the parental line. Although TK6 parental showed undetectable endogenous levels of DHFR, a faint band is evident in the MTX-resistant subline, demonstrating an increase in expression.

To determine if the increased levels of MSH3 and DHFR protein stemmed from an amplification event, relative amplification of the DHFR and MSH3 genes was determined in parental and corresponding MTX-resistant variant lines using a quantitative PCR method. As can be seen from Figure 1B, both DHFR and MSH3 were amplified ∼7-fold in the TK6/MTX cell line (P = 0.0058 and 0.0421, respectively, by two-tailed unpaired t-test). In the PreB697 cell lines, there was a 3-fold amplification of MSH3 in the resistant line (P = 0.071) but there was no difference in DHFR copy number between parental and MTX-resistant lines (P = 0.235). These results suggest that in the TK6/MTX line, increased protein levels of MSH3 and DHFR is due to co-amplification of the DHFR and MSH3 genes, whereas in the PreB697/MTX lines increased levels may be due to low-level amplification along with transcriptional/translational mechanisms.

In vitro MMR assay

To assess the impact of increased MSH3 protein levels in the MTX-resistant lines on MMR function, their ability to repair base–base mismatches and IDLs was determined using a functional assay for MMR. G-T and Δ5 substrates were used and the repair efficiencies are shown in Figure 2. No differences were seen between the repair of mismatched bases (G-T substrate) and IDLs (Δ5 substrate), when comparing each of the cells lines with the corresponding MTX-resistant sublines, suggesting that the increased expression of MSH3, due to gene amplification, is insufficient to perturb MMR proficiency.

Sensitivity to 6-TG and MTX

To determine the effect of increased MSH3 protein levels on thioguanine sensitivity, both pairs of parental and MTX-resistant cell lines were assayed for sensitivity to the thiopurine drug, to 6-TG and also to MTX, which served as a positive control. Data are shown in a supplemental figure. There was no difference in sensitivity of the cell lines to 6-TG, with EC50 values of 265 nM for TK6, 302 nM for TK6/MTX, 1 μM for PreB697 and 1.1 μM for PreB697/MTX. In contrast, the EC50 values for MTX were 17 nM for TK6, 100 nM for TK6/MTX, 12 nM for PreB697 and 89 nM for PreB697/MTX, demonstrating a 5- to 7-fold increased resistance of the variant lines (P < 0.0001, one-way analysis of variance).

Analysis of patient samples for DHFR/MSH3 co-amplification

To assess the levels of gene amplification in primary samples, a cohort of childhood ALL leukaemia samples, 22 at presentation and 47 at relapse, were assayed for MSH3 and DHFR gene amplification. Of the 47 relapse samples, 17 had matched presentation samples, allowing paired analyses. In addition, 20 normal DNA samples extracted from peripheral blood mononuclear cells from volunteers within the laboratory were similarly analysed as controls for normal copy number. Results are shown in Figure 3A. For the DHFR gene, the mean values for the three groups were found to be significantly different with the mean values of 1.25, 0.988 and 0.753 for presentation, relapse and normal, respectively (P = 0.0031, one-way analysis of variance test). By establishing an upper limit of 1.2 for normal DHFR copy number based on the maximum value for normal samples, 27% (six from 22) of presentation samples and 30% (14 from 47) at relapse were thus deemed to have >2 DHFR gene copies. Only two patients showed a gene amplification level approaching that seen in the cell line models. There was no difference in the MSH3 mean values between normal, presentation and relapse cohorts (P = 0.5059) and only two from 21 (9.5%) presentation and two from 47 (4.3%) relapse patients had values indicative of >2 gene copies. From these four, three samples from two patients had coincident increased DHFR gene copy levels with one patient having values of 1.45 and 2.034 for DHFR and MSH3 levels at presentation and 1.302 and 4.008 at relapse, respectively. In only this patient did MSH3 gene copy approach that seen in the MTX-resistant cell lines. Figure 3B shows data for the 17 matched pairs. There was no statistical difference between the paired presentation and relapse DHFR gene levels (P = 0.517, paired t-test). Two cases showed a reduction at relapse of 3- and 4-fold and one showed a 3-fold increase at relapse. Similarly for MSH3, there was no significant difference in paired MSH3 gene levels (P = 0.184, paired t-test).

Taken together, these results fail to show any evidence for DHFR and MSH3 co-amplification to a degree that would impinge on MMR function either before or after prolonged MTX/thiopurine therapy, although low-level co-amplification of both is apparent in some cases at presentation. However, about a third of cases at presentation and at relapse show evidence for low-level DHFR amplification alone.

RQ-PCR analysis of MSH3 messenger RNA levels in cell lines and patient samples

To compare MSH3 messenger RNA (mRNA) levels in the MTX-resistant sublines with those in clinical samples, a quantitative real-time PCR was employed. mRNAs extracted from bone marrow blasts obtained from 31 ALL patients at presentation and 17 at relapse were available for analyses. There was no material available from matched pairs. A series of leukemic cell lines were similarly analysed to determine the variation in MSH3 mRNA expression with different immunophenotypes. The data are shown in Figure 4. All the leukemic cell lines showed similar levels of MSH3 mRNA but the MTX variants of TK6 and PreB697 had ∼7-fold higher expression (P < 0.01, two-tailed unpaired t-test). In the clinical samples, there was a higher expression of MSH3 seen at relapse compared with the

Fig. 2. Bar charts showing base–base repair (G-T) and IDL repair (Δ5 bases) in TK6, PreB697 and their MTX-resistant sublines. Mean and SEM are shown from three independent experiments.
presentation group ($P = 0.0224$, two-tailed unpaired $t$-test). However, in only one patient did the MSH3 mRNA reach that seen in the MTX-variant sublines, but this was not associated with increased MSH3 copy number.

**Discussion**

In this study, we set out to determine whether concomitant amplification of the MSH3 gene, as a by-product of DHFR amplification, was likely to dysregulate the MMR pathway and whether it occurred in primary samples from children with ALL, either at diagnosis or after prolonged MTX therapy. Firstly, we generated MTX-resistant cell lines from a B-lineage ALL cell line and also from a non-neoplastic, lymphoblastoid cell line, by culturing under increasing concentrations of MTX, up to a maximum of 40 nM. We show that MTX-resistant variants possessed higher DHFR and MSH3 protein levels than in the TK6 cell lines was attributable to co-amplification of DHFR and MSH3 as demonstrated by a 7-fold increase in copy number of both genes. In the PreB line, although there was evidence for increased MSH3 copy number, DHFR copy number was apparently unchanged. It is possible that the increased protein level in this cell line is due to low-level amplification in combination with additional, transcriptional alterations. For example, the transcription factors E2F1 and Sp1 have been shown to act synergistically to activate DHFR (20,21) and E2F1 has been shown to transcriptionally activate other MMR proteins, MSH2, MSH6 and PMS2 (22), therefore it could be that common transcription factors may act to upregulate the expression of these two genes. Nevertheless, although the increased DHFR levels were associated with increased MTX resistance, the increased MSH3 protein levels were not sufficient to perturb MutSα function, in

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**Fig. 3.** Scatter plots showing relative amplification of (A) DHFR and MSH3 genes in ALL patients at presentation, relapse and normal individuals and (B) in the presentation/relapse paired samples for DHFR and MSH3.

**Fig. 4.** (A) Relative quantitation of MSH3 mRNA in parental TK6 and PreB697 and MTX-resistant sublines along with a series of leukaemic cell lines. Error bars show mean and SEM from three independent assays. (B) Scatter plot showing relative mRNA expression of MSH3 in ALL patients at presentation and relapse.
terms of either MMR capability or thioguanine sensitivity. This is in contrast to earlier studies that showed MMR deregulation in a MTX-resistant variant of the acute promyelocytic cell line, HL-60 that was attributable to co-amplification of MSH3 and DHFR (9,10). However, this cell line was generated under considerable selection pressure with concentrations of MTX reaching 1 μM and consequently resulted in significant amplification of DHFR and MSH3, with a reported 200 copies per cell. Our study generated a MTX resistant, ALL cell line using a MTX concentration estimated to be the sustained, pharmacological level achieved in children undergoing MTX-based, maintenance therapy (15) and thus may explain this disparity.

Secondly, we investigated DHFR/MSH3 co-amplification in clinical samples. We found no evidence for significant levels of co-amplification in a large cohort of samples taken at disease relapse after MTX therapy that lasts for 2–3 years, depending on gender. Nor did we find evidence for it in a smaller cohort of diagnostic samples. However, low-level amplification of both genes was apparent in diagnostic samples from two patients and in one was still evident in the paired relapse sample. In fact, MSH3 copy number increased at relapse to a similar level seen in the MTX subline levels and interestingly, analyses of the matched diagnostic sample suggested that copy number had doubled at relapse, suggestive of an amplification event induced by chemotherapy. However, the increased copy number in this patient was not associated with a parallel increase in MSH3 at the mRNA level.

We also investigated MSH3 at the transcriptional level and showed a small (1.6-fold) but significant increase in the mean MSH3 mRNA levels at relapse, with one patient expressing levels similar to those seen in the MTX resistance cell line models but this was not due to an increase in MSH3 copy number. However, even in this patient, data from the MTX-variant cell lines show that this relatively high level of MSH3 is unlikely to impinge on MMR function.

A number of studies have assessed the incidence of MMR defects in haematological malignancies (19,23–30). However, although we and others have found a surprisingly high incidence of MMR defects in leukemic cell lines (19,31,32), with the exception of therapy-related leukemias (33), the incidence in de novo leukemias is rare. Most studies rely on microsatellite instability analysis as a functional marker of MMR, but sequestration of MSH6 by overexpression of MSH3 may lead to a phenotype akin to that of MSH6 mutant cells that do not have the classical microsatellite instability associated with MLH1 or MSH2 mutant tumours and thus may make detection more difficult (34). Thus, although this study rules out MSH3 overexpression as a mechanism for MSH2 sequestration and MutSx deficiency in childhood ALL, it may occur in other malignancies, particularly as an acquired event in chemotherapeutic regimens employing high-dose MTX.

MTX plays a major role in the treatment of several types of malignancy, including childhood ALL. The two most common causes of resistance to this drug both in vivo and in vitro models are decreased influx into the cell due to alterations in the reduced folate carrier and increased DHFR activity due to amplification of the gene or increased translation (1). In this study, we confirm the findings of Goker et al. (2) and show that low-level amplification of DHFR is apparent in both presentation and relapse samples. From our paired analyses of DHFR levels, increase in copy number was not a consistent feature of relapse after MTX exposure. Interestingly, in the Goker study, of three paired samples, only one showed an increase in DHFR copy number at first relapse, whereas the remaining two showed an increase in samples taken at second relapse. Our cohort of 47 relapse samples consisted principally of those of first relapse but the time to relapse and the cumulative MTX exposure may have a bearing on increased DHFR gene copy number as a mechanism of MTX resistance.

In summary, we show that co-amplification of both MSH3 and DHFR can be induced by a pharmacologically relevant dose of MTX in lymphoid cell lines but not to a level that impacts on MMR functionality, in terms of MMR capacity or 6-TG sensitivity. In addition, we find no evidence for significant co-amplification of these genes in primary ALL samples, either de novo or at relapse after prolonged MTX therapy.

Supplementary data

Supplementary data are available at Carcinogenesis’s Online.

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Conflict of Interest Statement: None declared.

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