

Characterization of *ABCG2* gene amplification manifesting as extrachromosomal DNA in mitoxantrone-selected SF295 human glioblastoma cells

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Abstract

The human *ABCG2* gene, located on chromosome 4, encodes an ATP-binding cassette half-transporter that has been shown to confer resistance to chemotherapeutic agents. Relatively little is known about the mechanisms controlling expression of *ABCG2*. In previous studies, we had shown that overexpression of *ABCG2* can result from rearrangement or gene amplification involving chromosome 4. To better characterize the mechanisms of *ABCG2* overexpression, SF295 glioblastoma cells were exposed to increasing amounts of mitoxantrone to generate the SF295 MX50, MX100, MX250, and MX500 sublines, maintained in mitoxantrone concentrations ranging from 50 to 500 nmol/L. Northern blot analysis confirmed overexpression of *ABCG2* mRNA, and immunoblot analysis demonstrated increased protein expression in the selected cell lines. Efflux of BODIPY-prazosin confirmed a functional protein. *ABCG2* gene amplification was observed in all resistant sublines, as determined by Southern blot analysis. Fluorescence in situ hybridization (FISH) revealed amplification of *ABCG2* via double minute chromosomes (dmns) detected in metaphase chromosome spreads in the SF295 MX50 and MX100 sublines. At higher levels of drug selection, in the MX250 and MX500 sublines, fewer dmns were observed but homogeneously staining regions (hsr) were visible with FISH analysis, revealing reintegration of the *ABCG2* gene into multiple chromosomes. Spectral karyotyping (SKY) demonstrated multiple clonal and nonclonal rearrangements of chromosome 4, including hsrs. These results suggest that amplification of *ABCG2* occurred initially in the form of dmns, followed by chromosomal reintegration of the amplicon at multiple sites. This occurred with increasing drug-selection pressure, generating a more stable genotype. © 2005 Elsevier Inc. All rights reserved.

1. Introduction

Host and tumor genetic alterations, epigenetic changes and tumor microenvironment may all contribute to failure of cancer chemotherapy due to multidrug resistance [1]. In the central nervous system, both cellular mechanisms and the blood–brain barrier may combine to prevent the achievement of adequate and sufficiently sustained levels of exposure to cytotoxic agents, leading to the failure of systemic chemotherapy of malignant brain tumors. Overexpression of

members of the ATP-binding cassette (ABC) transporter family of proteins has been widely studied as a mechanism of resistance in cancer. Increased expression of MDR-1/P-glycoprotein has been suggested as a cause of chemotherapy failure in malignant gliomas and in other malignancies [1].

A gene belonging to the G subfamily of ABC transporters, *ABCG2* (alias *MXR*, *BCRP*, and *ABCP1*), encodes an ABC half-transporter protein consisting of 655 amino acids with a potential role in resistance to chemotherapy agents including mitoxantrone and camptothecins [2–4]. The *ABCG2* gene, spanning 66 kb, is located at chromosome band 4q22 [5] and consists of 16 exons and 15 introns [6]. Relatively little is known about the mechanisms controlling expression

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of ABCG2. In previous studies with in vitro cell-line models [5], we have shown that overexpression of ABCG2 occurs due to gene amplification, as well as due to rearrangement involving chromosome 4.

In the present study, we demonstrate amplification of ABCG2 via double minute chromosomes (dmns) in mitoxantrone-selected SF295 malignant glioma cells. The dmns reintegrate into the genome with increasing selection pressure and manifest as homogeneously staining regions (hsr) in multiple chromosomes. Although reintegration of the dmns has been postulated in several model systems, it has rarely been documented. We surmise that this occurred as a consequence of increasing drug-selection pressure, generating a more stable genotype.

2. Materials and methods

2.1. Cell lines

The human malignant glioma cell line SF295 was selected in increasing concentrations of mitoxantrone to yield the SF295 MX50, MX100, MX250, and MX500 cell lines, maintained in 50, 100, 250, and 500 nmol/L mitoxantrone, respectively. All selected cell lines were maintained in constant culture with mitoxantrone for at least 3 months before cytogenetic analysis. The parental line and resistant sublines were grown in RPMI supplemented with 10% fetal calf serum (FCS) with penicillin–streptomycin and glutamine. The ABCG2-overexpressing subline S1-M1-80, maintained in RPMI with 80 μ mol/L mitoxantrone, was used as a positive control.

2.2. Flow cytometry

Flow cytometry-based studies using BODIPY-prazosin as a fluorescent substrate were performed as previously described [7]. Briefly, a suspension of log-phase cells was obtained using trypsinization. Cells were incubated in 250 nmol/L BODIPY-prazosin (Molecular Probes, Eugene, OR) with or without 100 μ mol/L fumitremorgin C (FTC) in complete medium (phenol red-free improved minimum essential medium [IMEM, Richter's medium] with 10% FCS) at 37°C in 5% CO₂ for 30 minutes. FTC has been shown to be a specific blocker of ABCG2 [8]. Subsequently, the cells were washed once with cold complete medium and then resuspended in complete medium continuing with or without 100 μ mol/L FTC for a 60-minute efflux period at 37°C. Cells were then washed in ice-cold Dulbecco's phosphate-buffered saline and kept on ice in the dark until flow cytometric analysis. A FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 488-nm argon laser and a 530-nm bandpass filter was used to detect BODIPY-prazosin. At least 10,000 events were collected. Debris was eliminated by gating on forward versus side scatter and dead cells were excluded based on propidium iodide staining.

2.3. Immunoblot analysis

Immunoblot analysis was performed as previously described [9]. Briefly, microsomal membrane protein was subjected to gel electrophoresis and transferred onto nitrocellulose membranes; the blots were probed with the monoclonal antibody BXP-21 [10]. A secondary antibody conjugated to horseradish peroxidase was then applied for 1 hour, after which the blot was subjected to enhanced chemiluminescence detection (SuperSignal; Pierce Chemical, Rockford, IL).

2.4. Northern blot analysis

RNA was extracted from parental and resistant cells using RNA-STAT 60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. Northern blot analysis was performed as previously described [11], using a riboprobe generated from the first 662 bp of ABCG2 subcloned into a pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA).

2.5. Southern blot analysis

DNA from all cell lines was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Ten micrograms of DNA were then digested with *Hind*III, separated on a 1% agarose gel, and transferred to nitrocellulose. The blots were then hybridized with full-length ABCG2 2.0 kb cDNA at 42°C in Hybrisol I (Intergen, Purchase, NY). The ABCG2 probe was labeled with the Prime-It RmT random primer labeling kit (Stratagene, La Jolla, CA) and [³²P]dCTP (3,000 Ci/mol) (Amersham Biosciences, Piscataway, NJ). Blots were washed in 5× standard saline citrate (SSC) for 15 minutes at room temperature, followed by two washes in 1× SSC–0.1% SSC preheated to 42°C for 15 minutes each. Autoradiographs were obtained after 24 hours at –70°C.

2.6. Fluorescence in situ hybridization analysis

Metaphases from cell lines were harvested using the mitotic arresting agent colcemid (100–500 ng/mL) followed by hypotonic treatment with 0.04 mol/L KCl and 0.5% sodium citrate, with subsequent fixation of the cell pellet in methanol–acetic acid (3:1). Fluorescence in situ hybridization (FISH) analysis was performed using whole chromosome paint kits for chromosome 4 in SpectrumGreen (Vysis, Downers Grove, IL) and an ABCG2 bacterial artificial chromosome (BAC) probe (>50 kb and containing the 3' end of the gene; NCBI Clone Registry ID RP11-368G2, Invitrogen, Carlsbad, CA) to localize the ABCG2 gene to normal and rearranged chromosomes. The BAC hybridization procedure used is similar to other FISH technologies. The ABCG2 BAC probe was labeled by nick translation using biotin-16 dUTP or digoxigenin-11 dUTP. The ABCG2 BAC probe localizes to chromosome position 4q22 and has previously been used to study ABCG2 overexpression [5]. All slides

were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

2.7. Spectral karyotyping analysis

Spectral karyotyping (SKY) was performed according to the technique of Schröck et al. [12] and Macville et al. [13] for identification of all chromosomal abnormalities in the cell lines. Images were acquired with a spectral cube system (Applied Spectral Imaging, Migdal Ha'emek, Israel) attached to an epifluorescence microscope (DMRXA; Leica, Wetzlar, Germany), and the emission spectrum was measured with a custom-made triple-band-pass filter

(Chroma Technology, Brattleboro, VT). Both probe and DAPI images were acquired.

3. Results

3.1. Overexpression and amplification of ABCG2 in SF295 cells selected in mitoxantrone

To confirm expression of ABCG2 in the mitoxantrone-selected SF295 sublines, Northern blot analysis of RNA from the parental and selected cells was performed; this revealed increasing ABCG2 expression at the RNA level with increasing mitoxantrone concentration (Fig. 1A). RNA

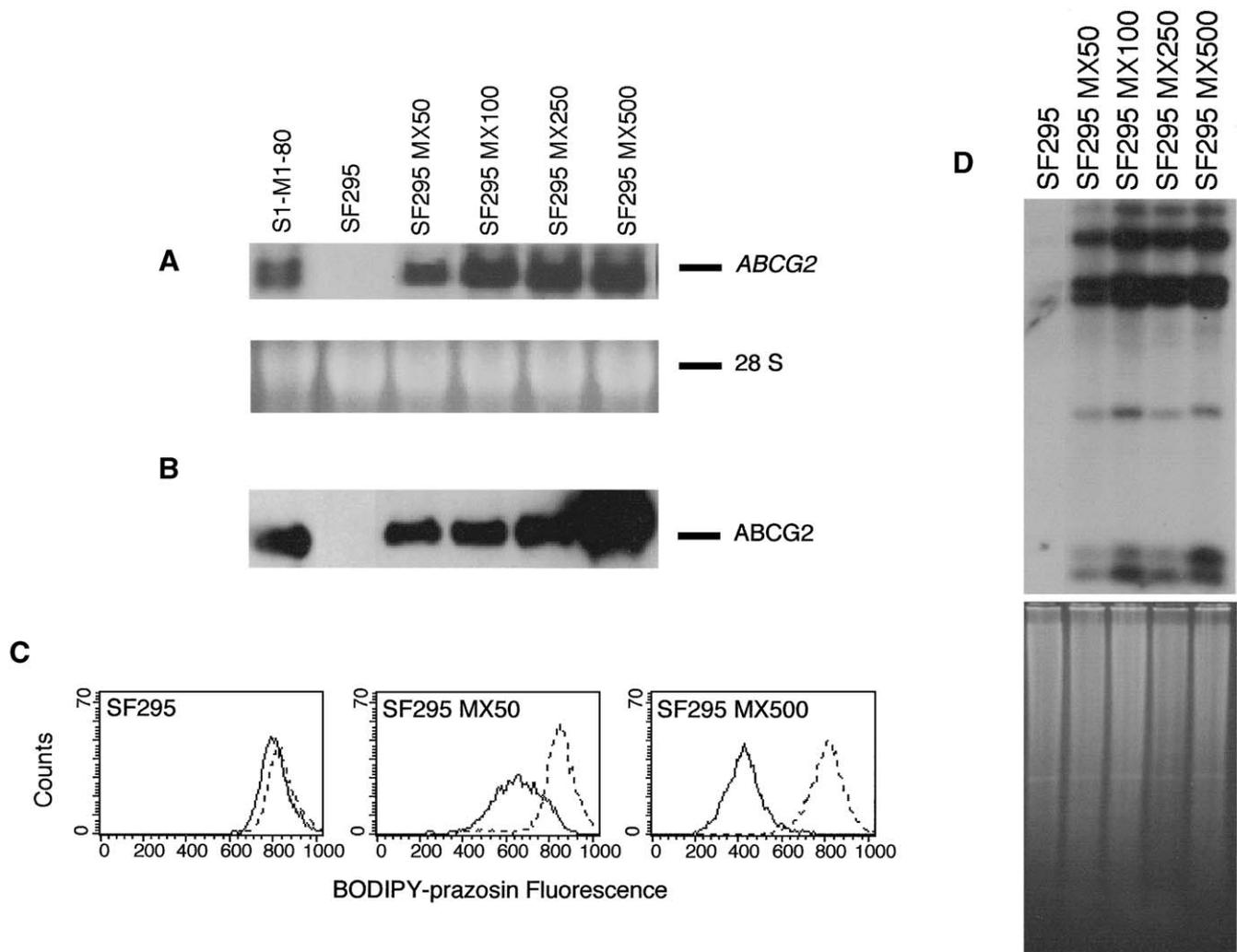


Fig. 1. Overexpression of ABCG2 in mitoxantrone-selected SF295 cells. (A) Northern blot analysis of RNA from SF295 parental and mitoxantrone-resistant sublines using a riboprobe generated from the first 662 bp of ABCG2. ABCG2-overexpressing S1-M1-80 cells are included as a positive control. (B) Western blot analysis of protein extracted from SF295 parental cells and resistant sublines probed with the anti-ABCG2 antibody BXP-21. Protein from S1-M1-80 cells is included as a positive control. (C) Cells were incubated in 250 nmol/L BODIPY-prazosin with or without 10 μmol/L FTC, washed, then allowed to efflux in prazosin-free media continuing with (dotted line) or without (solid line) FTC. Representative results for SF295 parent, MX50, and MX500 cells are shown. (D) Southern blot analysis of parental and resistant sublines. Genomic DNA from SF295 parental, MX50, MX100, MX250, and MX500 cells was digested with *EcoRI*, separated on a 1% gel and transferred to nitrocellulose. The blot was probed with random primer-labeled ABCG2 cDNA. Amplification of ABCG2 was detected in all selected cell lines.

obtained from S1-M1-80 cells, previously shown to overexpress ABCG2, was included as a positive control. Immunoblot analysis of membrane protein extracted from SF295 parental and resistant sublines probed with the anti-ABCG2 antibody BXP-21 is shown in Fig. 1B. S1-M1-80 cells were again included as a positive control. Increased ABCG2 expression was observed in all mitoxantrone-selected cells with highest levels found in the MX500 subline.

Efflux assays with BODIPY-prazosin were performed on parental and mitoxantrone-selected SF295 cells to confirm expression of a functional ABCG2 protein. Intracellular BODIPY-prazosin fluorescence was subsequently quantitated on a flow cytometer. We have previously shown that the difference between the efflux peak with FTC and the efflux peak without FTC (termed the *FTC-inhibitable efflux*) is a functional measure of ABCG2 and is proportional to its expression [7]. SF295 parental cells display little FTC-inhibitable BODIPY-prazosin efflux, as evidenced by the small difference between the two curves, suggesting a low but detectable level of ABCG2 expression. In SF295 MX50 and MX500 cells, however, increasing levels of FTC-inhibitable efflux (greater distance between the curves in Fig. 1C)

are observed with increasing concentrations of the selecting drug and are consistent with increased protein expression.

Southern blot analysis of DNA extracted from drug-selected sublines SF295 MX 50, 100, 250, and 500 showed amplification of the *ABCG2* gene. Digestion with *Hind*III revealed three major fragments. Compared to the level of *ABCG2* found in parental cells, shown above to be triploid, the MX50 cells demonstrated a three- to fivefold increase in intensity and MX 100, 250, and 500 demonstrated a six- to eightfold increase in *ABCG2* copy number, relative to the parental cell line. No evidence of gene rearrangement was detected by Southern blot analysis (Fig. 1D).

3.2. FISH analysis of selected cell lines

To explore the mechanisms of *ABCG2* amplification in the SF295 series of cell lines, FISH studies were performed on metaphase preparations of the parental and resistant sublines. Parental SF295 cells displayed a triploid karyotype with the *ABCG2* BAC probe localized to three copies of the normal chromosome 4 (Fig. 2A). In the drug-resistant SF295 MX50 subline, extrachromosomal DNA was observed by DAPI staining. FISH analysis demonstrated the

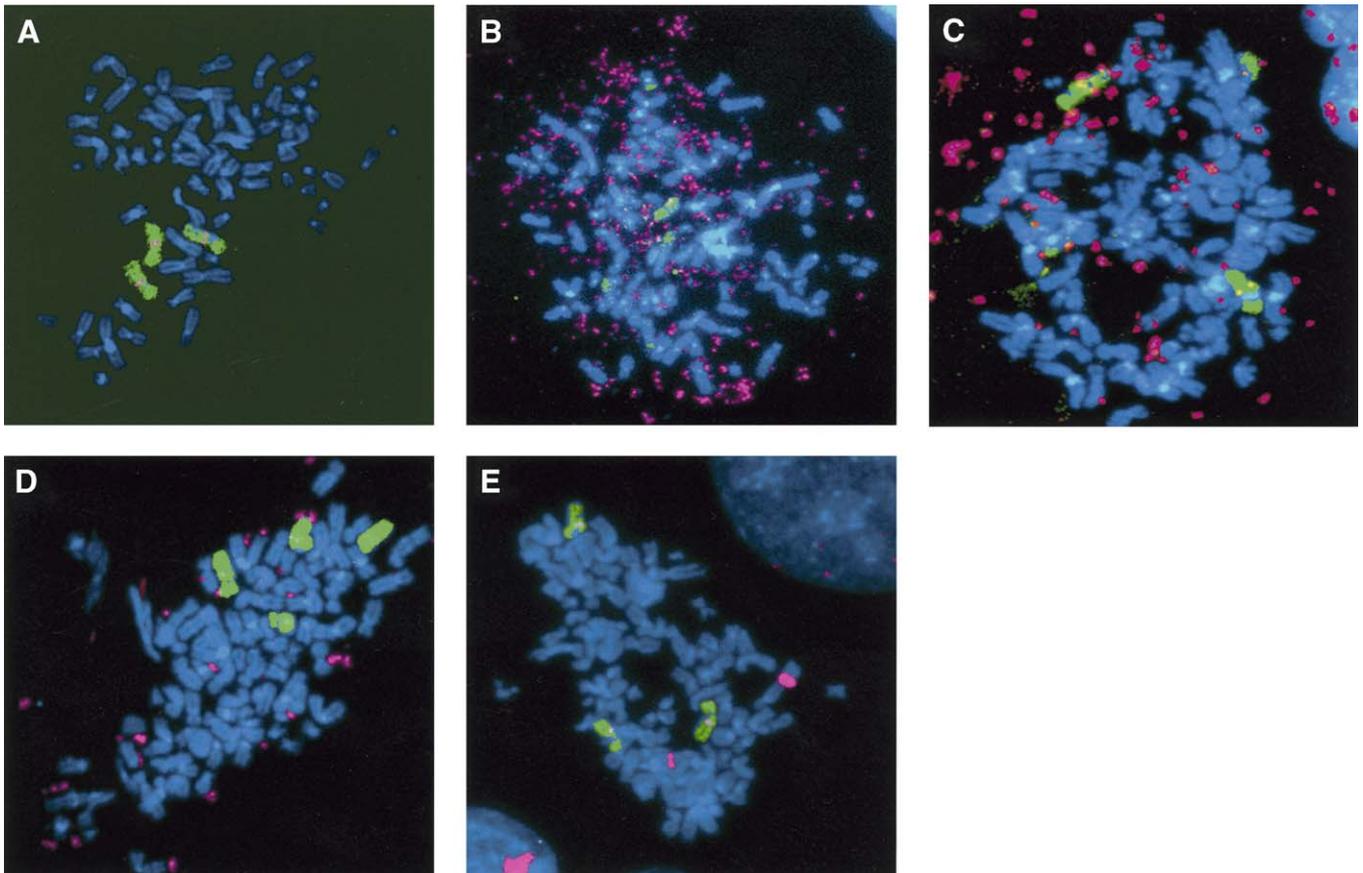


Fig. 2. FISH analysis of SF295 parental and mitoxantrone-resistant sublines. Cytogenetic preparations of SF295 parental (A), MX50 (B), MX100 (C), MX250 (D), MX500 (E) cells were subject to FISH analysis with whole chromosome 4 paint (green) and an *ABCG2* BAC probe (magenta). Chromosomes were counterstained with DAPI.

extrachromosomal DNA to be dmms containing amplified sequences of *ABCG2* as evidenced by colocalization with the *ABCG2* BAC probe (Fig. 2B). With increasing selection pressure, fewer yet larger dmms were observed, as shown in the MX100 (Fig. 2C) and MX250 (Fig. 2D) FISH images. In the MX500 subline, a loss of dmms was observed, coupled with evidence of reintegration of the amplified sequences into the genome, as shown by the hrs (magenta in Fig. 2E). In approximately half of the cells examined in the MX500 subline, the length of the integrated amplicon containing the *ABCG2* gene was more pronounced, suggesting two different clones. Representative results from 50 metaphases are shown in Fig. 2; at least three independent FISH analyses were performed for each cell line.

3.3. SKY analysis of mitoxantrone-selected cells

Spectral karyotyping (SKY) was subsequently performed on SF295 parental, SF295 MX250, and SF295 MX500 metaphase spreads to confirm reintegration of the dmms into the genome. The SKY results are summarized in Table 1 and representative results are shown in Fig. 3. Both the parental line and the two drug-resistant sublines showed considerable variation in chromosome number per cell. The number of structural abnormalities (translocations, insertions, and deletions) ranged from a few in the SF295 parental line (and none of them involving chromosome 4) to many in the MX500 subline. SF295 MX250 cells also showed only normal copies of chromosome 4, with the exception of one cell, which showed a t(4;20) translocation (Fig. 3, middle panel); the dmms seen by FISH in MX250 were not detectable in the SKY preparation. FISH analysis of the MX500 subline suggested reintegration of the dmms containing the *ABCG2* amplicon, and this was supported by the SKY results, which showed the presence of clonal and nonclonal markers involving the insertion of segments of chromosome 4 into complex markers, particularly der(1)t(1;4), der(1)t(1,4,15,?), and der(2)t(2,4,15,22). The increase in aneuploidy, particularly the cell-to-cell variation in the clonal markers, suggests a further loss of fidelity in DNA replication with increasing drug selection.

4. Discussion

We generated *ABCG2*-overexpressing sublines by exposing the SF295 cell line to increasing concentrations of mitoxantrone. SF295 cells have been shown to express low but detectable levels of *ABCG2* [7]. The evolution of the phenotype with increasing mitoxantrone selection showed varying degrees of overexpression of the *ABCG2* gene and protein, confirmed by Northern blot and immunoblot analysis. FISH analysis of earlier steps of the SF295 cells selected in mitoxantrone showed amplification of *ABCG2* manifested first in the form of dmms. Further characterization of chromosomal aberrations involving the *ABCG2* gene on chromosome 4 was performed using FISH and SKY analysis of SF295 parental as well as drug-selected cells.

Development of acquired resistance to multiple chemotherapeutic drugs is thought to be a common reason for failure of treatment of many cancers in general and brain tumors in particular. One of the means by which cells can become drug resistant is by amplification of specific genes implicated in conferring the resistant phenotype. The overexpression of an ABC transporter is a phenotypic feature often observed in cancer cells selected for drug resistance in vitro. The sublines studied here afford insights into the mechanism of amplification of the *ABCG2* gene. Overexpression of a functional *ABCG2* protein was also demonstrated in parallel experiments.

The most intriguing finding of this phenotypic study was the appearance and persistence of clones of cells with dmms at early steps of selection that randomly reintegrated into the euchromatin of multiple chromosomes with increasing drug-selection pressure, while leading to the gradual disappearance of dmms. Currently, dmms are understood to be packages of amplified genetic material that have separated from a chromosome and exist and autoreplicate as extrachromosomal bodies within the nucleus. It has been proposed that submicroscopic, rapidly inducible DNA molecules (termed *episomes*) that multimerize over time are the precursors of dmms [14–16]. The other manifestation of gene amplification is a *homogeneously staining region*, a descriptive term based on the uniform appearance of an amplified region in a G-banded chromosome.

Table 1

Summary of chromosomal aberrations involving chromosome 4 as revealed by spectral karyotyping (SKY) of glioblastoma cell line SF295 and its drug-selected sublines

SF 295	Number of cells analyzed	Normal chromosome 4, modal number (cell ploidy)	Number of cells with translocations involving chromosome 4	Abnormality	Found in <i>n</i> cells
Parental: Clone 1	8	2–3 (near-triploid)	0		
Parental: Clone 2	3	5–6 (near-hexaploid)	0		
SF295 MX250	4	1–3 (triploid-tetraploid)	1 (1 cell only)	der(4)t(4;20)(q12;?)	1
SF295 MX500	8	1–3 (triploid-tetraploid)	1–2 clonal/cell	der(1)(4qter→4q11::1p?→1q?:4q?)	3
				der(1)(15?:1p?→1q?:15?:4?:?)	3
				der(2)(2pter→2q11::4?:22?:15?:22?:4?)	5
			0–1 nonclonal/cell	der(4)t(4;17)	1
				der(7)t(4;7)	1
				der(8)t(4;8)	1



Fig. 3. Spectral karyotyping analysis of SF295 parental, MX250, and MX500 cells (classification colors). Top panel: Near-triploid parental cell with three normal copies of chromosome 4; Middle panel: Near-tetraploid MX250 cell with three normal and one abnormal copy (arrow) of chromosome 4; Lower panel: Near-triploid MX500 cell with one normal chromosome 4 and two clonal (large arrows) and one nonclonal (small arrow) translocations involving chromosome 4.

The exact method by which dmms are formed has yet to be elucidated. One theory suggests that extrachromosomal amplicons of circular DNA from a single precursor result from the postreplicative excision of a chromosomal fragment [17]. Another possibility is that multiple double-strand breaks occur within a preexisting hsr by way of fragile site activation [18,19]. Our data suggest that the postreplicative model explains the formation of dmms, because neither

rearrangements involving chromosome 4 nor losses of chromosome 4 are observed in the earliest step of selection at which dmms were observed (MX50). Additionally, we did not observe an initial hsr or evidence of hsr degradation in an earlier step of drug selection (SF295 MX20; data not shown). Reintegration of dmms into the genome and subsequent hsr formation has been suggested by others [17,20] and was confirmed in this study.

Many human cancer cell lines acquire dmns containing amplified drug-resistance genes, including multidrug resistance/P-glycoprotein (*MDR*; now *ABCB1*), multidrug resistance associated protein (*MRP*; now *ABCC1*), and dihydrofolate reductase (*DHFR*) [21–23]. Both dmns and hrs have been demonstrated as cytogenetic manifestations of *MDR* gene amplification in studies of in vitro selected cell lines [24]; however, amplification of known drug-resistant genes in clinical samples has not been convincingly demonstrated, other than for the extensively studied *DHFR* gene [25]. On the other hand, the occurrence of dmns containing genes involved in oncogenesis is well described in clinical samples. The *MYC* gene is the most commonly reported gene to be amplified in dmns in hematologic malignancies, followed by the *MLL* gene during disease progression [26–29]. In addition to genes involved in the hematologic malignancies acute myeloid leukemia (AML) and secondary myelodysplastic syndrome (MDS), other cancer-related genes, including *RAS*, *ERBB2* (alias *HER2*), *MYC*, *MDM2*, and *MYB* have also been shown to be amplified as dmns in solid tumors such as gastric adenocarcinoma [30], salivary gland tumors [31], and neuroblastoma [27].

Weak evidence has linked the emergence of dmns to drug resistance [19]. Fusion transcripts involving the *MLL* gene and *PAX7-FOXO1A* (alias *PAX7-FKHR*) in dmns have been reported during disease progression and development of drug-resistant tumors [16,32]. One report describes new amplification regions identified by FISH and SKY in nine patients with dmns in refractory hematologic malignancies, although the encoded sequences within them were not specifically characterized [14]. Finally, a clinical study confirmed that noncytotoxic doses of the drug hydroxyurea has the potential to eliminate dmns from cancer cells collected by abdominal paracentesis in advanced ovarian carcinoma, although a response to therapy was not observed [15]. Thus, although dmns are clearly implicated in oncogenesis, their presence in drug-resistant cancers is more obscure.

The detection of dmns in drug-selected cell lines can be viewed as evidence of a global defect in DNA repair. It is thought that genomic instability is enhanced by microsatellite instability, especially in the setting of defective mismatch repair genes. Thus, microsatellite instability associated with defective *TP53*, *CDKN2A* (alias *p16*), *PTEN*, and mismatch repair genes leads to genomic instability, and thence to various other somatic mutations in cancer in general and in glioma tumor cells in particular, including the derived cell line SF295 [33–36]. MMR (mismatch repair) deficient cancer cells that exhibit increased resistance to methotrexate also exhibit an increased frequency of chromosomal DNA integration, as assayed by transfection of linear DNAs into mammalian cells [37]. Thus, the present experiments also provide an indirect evidence of a defective mismatch repair system in the glioblastoma cell line SF295 that may have facilitated the chromosomal reintegration of amplified extra-chromosomal DNA, which in this case happened to be *ABCG2*. The marked aneuploidy that occurred with the step

to MX500 accompanied the reintegration of the *ABCG2* sequences and supports this hypothesis. Taken together, these results suggest that increasing drug resistance in the SF295 cells was accompanied by a progressive loss of mismatch repair capability.

These studies, showing the impact of increasing mitoxantrone selection on *ABCG2* overexpression in SF295 cells, offer insights into mechanisms that may underlie drug resistance in clinical oncology. The experiments suggest that reversal of drug resistance will ultimately require not only inhibition of ABC transporter function, but also prevention of the continued acquisition and retention of chromosomal abnormalities in cancer cells.

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