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BRIEF COMMUNICATION

Amplification of 4q21–q22 and the *MXR* Gene in Independently Derived Mitoxantrone-Resistant Cell Lines

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Molecular cytogenetic studies were conducted on three multidrug-resistant cancer sublines which are highly resistant to the chemotherapeutic agent mitoxantrone, an anthracenedione. The three independently selected sublines were derived by exposure to mitoxantrone or Adriamycin and do not overexpress *MDR1* or *MRP*. Two sublines, MCF-7 AdVp3000 and MCF-7 MX, showed an amplification peak at 4q21–q22, as demonstrated by comparative genomic hybridization (CGH), while the third, S1-M1–80, did not. FISH using a whole chromosome 4 paint demonstrated multiple rearrangements involving chromosome 4 in MCF-7 AdVp3000 and MCF-7 MX, while S1-M1–80 contained only a simple reciprocal translocation. The parental cell lines had no chromosome 4 rearrangements and no copy number gain or amplification of chromosome 4. Spectral karyotyping (SKY) analysis revealed a balanced translocation, t(4;17)(q21–q22;p13) in S1-M1–80 and multiple clonal translocations involving chromosome 4 in MCF-7 AdVp3000 and MCF-7 MX. A novel cDNA, designated *MXR*, which encodes an ABC half-transporter and is highly overexpressed in the three sublines, was localized to chromosome 4 by somatic cell hybrid analysis. Southern blot analysis demonstrated amplification of the *MXR* gene in MCF-7 AdVp3000 and MCF-7 MX, but not in S1-M1–80. FISH studies with a BAC probe for *MXR* localized the gene to 4q21–22 in the normal chromosome 4 and revealed in both MCF-7 AdVp3000 and MCF-7 MX amplification of *MXR* at one translocation juncture, shown by SKY to be t(4;5)(4qter→4cen→4q21–22::5q13→5qter) in MCF-7 AdVp3000 and t(6;4;6;3)(6pter→6q15::4q21–q22::hsr::6q?::3q?27→3qter) in MCF MX; neither of the breakpoints in the partner chromosomes showed amplification by CGH. The data are consistent with the hypothesis of a transporter, presumably that encoded by the *MXR* gene, mediating mitoxantrone resistance. The *MXR* gene encodes a half-transporter and the absence of cytogenetic evidence of coamplification of other regions suggests that a partner may not be overexpressed, and instead the *MXR* half-transporter homodimerizes to mediate drug transport. *Genes Chromosomes Cancer 27:110–116, 2000.* Published 2000 Wiley-Liss, Inc.†

Multidrug resistance has occupied a significant share of scientific investigation over the past decade. Overexpression of the membrane transporter P-glycoprotein (Pgp) and its encoding gene, *MDR1*, was originally cited as a potential explanation for the problem of clinical drug resistance and became a target for resistance reversal strategies (Juliano and Ling, 1979). However, reversal strategies with P-glycoprotein antagonists have had limited success (Sandor et al., 1998). Although the use of antagonists of inadequate potency is one reason for the limited success, the presence of other unidentified mechanisms of resistance, including additional drug transporters, has been proposed as an additional explanation.

Mitoxantrone is an anthracenedione that shows promise in the treatment of leukemia, lymphoma, breast cancer, and prostate cancer. It binds avidly to nucleic acids, intercalating between opposing DNA strands, and inhibits DNA and RNA synthesis. We have studied the phenotype and genotype of a series of independently selected multidrug-resistant cancer sublines which have in common a characteristically high resistance to mitoxantrone with cross resistance to adriamycin and other anthracyclines, and to topotecan: MCF-7 AdVp3000, MCF-7 MX, and S1-M1–80 (Dalton et al., 1988; Dietel et al., 1990; Taylor et al., 1991; Nakagawa et al., 1992; Yang et al., 1995; Lee et al., 1997). Although mitoxantrone is a Pgp substrate, none of these cell lines overexpresses *MDR1* or *MRP*, but

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they do have an energy-dependent reduction in the accumulation of mitoxantrone, as well as daunomycin, bisantrene, and topotecan. Energy-dependent rhodamine and daunomycin efflux can also be demonstrated in these cells.

Gene amplification associated with *MDR1* or *MRP* overexpression has been observed in numerous in vitro model systems (Shen et al., 1986; Cole et al., 1992). In previous studies (Mickley et al., 1997; Knutsen et al., 1998), we presented molecular and cytogenetic evidence, using molecular cloning, fluorescence in situ hybridization (FISH), and comparative genomic hybridization (CGH), supporting chromosome rearrangement as a mechanism for the overexpression of the multidrug resistance gene *MDR1*. In the present study, we applied the same techniques to demonstrate amplification and chromosomal rearrangements in cells resistant to mitoxantrone.

Three mitoxantrone-resistant sublines were characterized in the present study, including two that were independently derived (in two different laboratories) from the MCF-7 human breast cancer line. MCF-7 AdVp3000 cells were selected with adriamycin in the presence of verapamil to prevent increased expression of Pgp and are maintained in 3,000-ng/ml adriamycin plus 5-μg/ml verapamil (Chen et al., 1990). MCF-7 MX cells were selected in mitoxantrone in a stepwise manner and were most recently reselected in 600-nM mitoxantrone (Nakagawa et al., 1992). The third line, S1-M1–80, was derived from the S1 clone of the LS174 human colon cancer cell line by selection in mitoxantrone in a stepwise manner (Rabindran et al., 1998) and is maintained in 80-μM mitoxantrone (Miyake et al., 1999).

CGH was performed to identify potential sites of gene amplification. The procedure was a modification of the method described by du Manoir et al. (1993). Control DNA was obtained from the lymphocytes of a cytogenetically normal female and the test DNA was extracted from parental and drug-resistant tumor cells. Ratio images and profiles were achieved using the Applied Imaging Cytovision System (Tyne and Wear, United Kingdom). For each drug-resistant subline, results were compared to those obtained in the parental line. The most striking finding was the occurrence of amplification at the junction of chromosome bands 4q21–q22 in the MCF-7 AdVp3000 and MCF-7 MX cells. No such amplification was observed in the S1-M1–80 resistant cells (Fig. 1).

FISH studies were performed on cytogenetic preparations from the parent and resistant cell lines

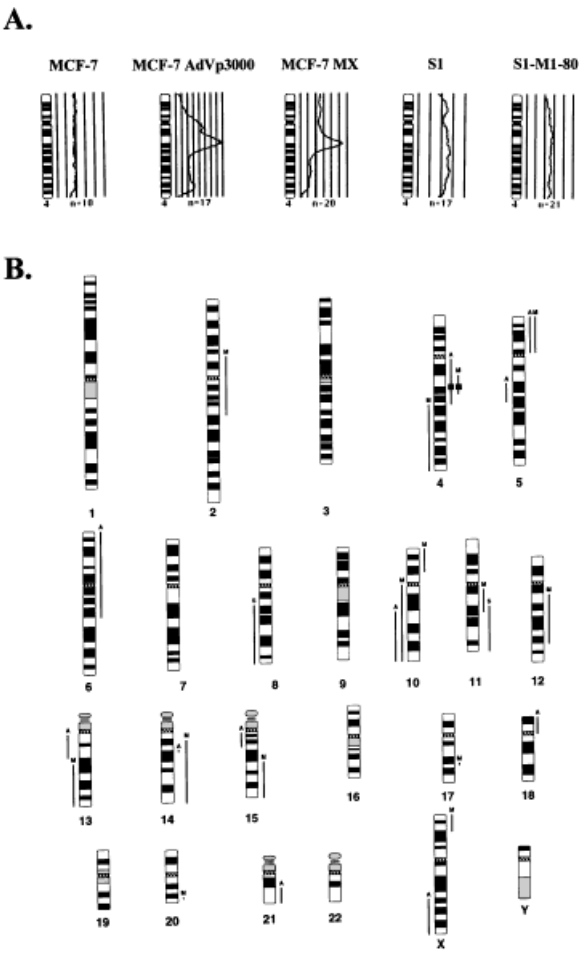

Figure 1. CGH in parent and drug-resistant tissue culture cell lines. **A:** DNA copy number changes in chromosome 4 for MCF-7 parent, MCF AdVp3000, MCF MX, S1 parent, and S1-M1–80. Note amplification peak at 4q21–q22 in MCF AdVp3000 and MCF-MX. **B:** Summary of DNA copy number changes in MCF AdVp3000 (A), MCF MX (M), and S1-M1–80 (S), each as compared to its respective parent line. Losses are shown on the left and gains on the right of each chromosome. Amplification sites are represented as solid squares or bars. Note amplification at 4q21–q22 in MCF AdVp3000 and MCF MX.

to evaluate the status of chromosome 4. FISH analysis with a whole chromosome paint 4 (WCP 4) Spectrum Green painting probe (Vysis, Downer's Grove, IL) revealed normal-appearing copies of chromosome 4 in the parent lines (three copies in the MCF-7 cell line, which has a modal chromosome number in the triploid range, and two copies in the diploid S1 cell line) and structural rearrangements in all the resistant lines. MCF-AdVp3000 and MCF MX, both of which showed amplification at 4q21–q22, had multiple rearrangements involving chromosome 4, while S1-M1–80 showed only one rearrangement suggestive of a balanced translocation.

Spectral karyotyping (SKY) (Schröck et al., 1996) was performed to identify all chromosomal rear-

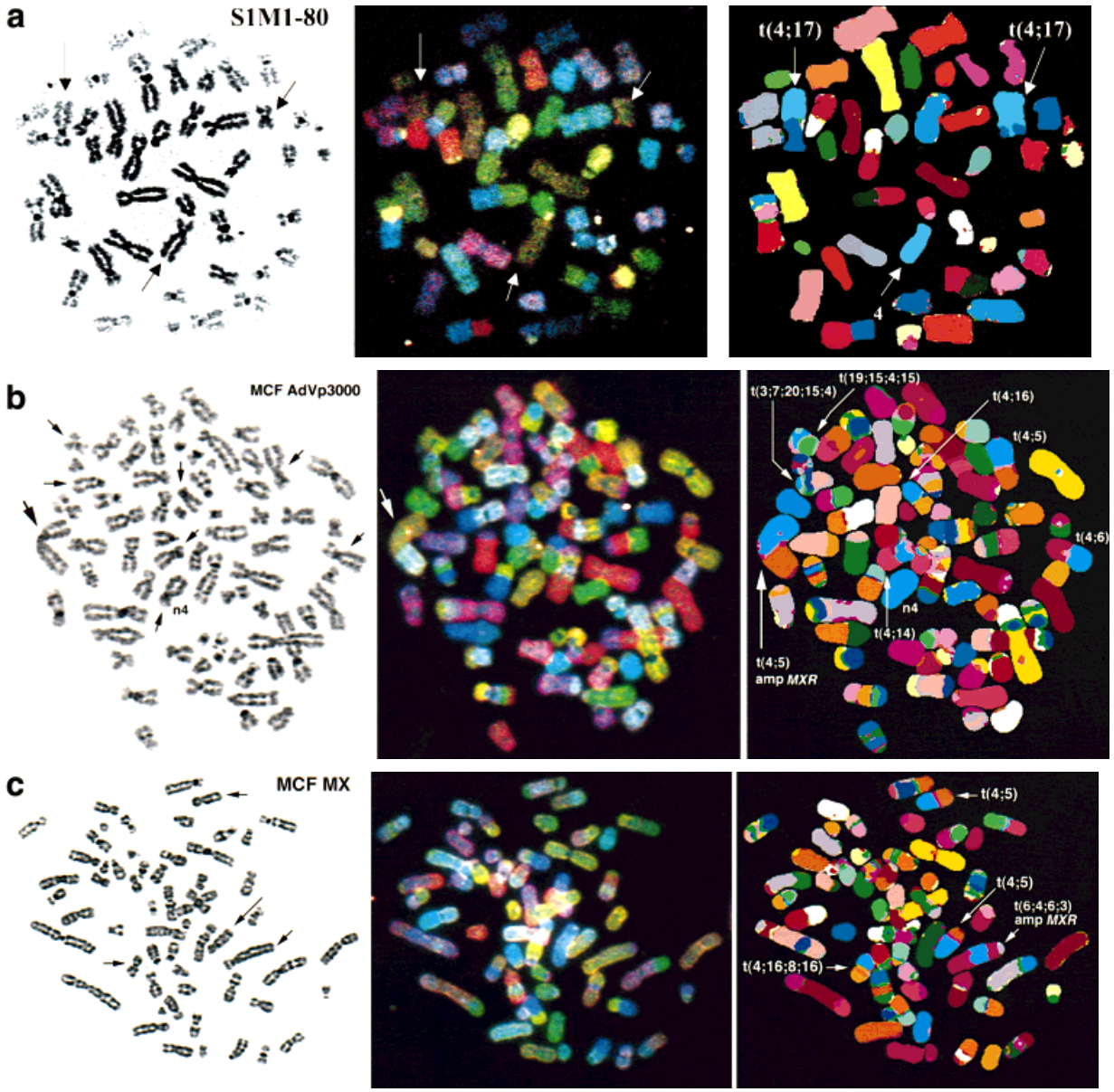

Figure 2. SKY analysis. A representative metaphase spread hybridized with SKY probes is depicted for each drug-resistant cell line. Left, inverse-DAPI image; middle, SKY display image; right, classified image. The clonal rearrangements are identified on the classified images and marked by arrows on the DAPI image; arrows in the SKY display images indicate the *MXR* rearrangement and amplification. Note that SKY uses combinatorial labeling with five different fluorochomes, one to four fluorochromes/chromosome; in some instances, additional colors at translocation interfaces do not reflect an additional chromosome segment but are the result of a blending of fluorochromes from chromosomes flanking the translocation breakpoint. **a:** S1-M1–80, t(4;17)(q21–q22;p13). **b:** MCF AdVp3000, t(4;5)(4qter→4cen→4q21–22::5q23→5qter). **c:** MCF MX, t(6;4;6;3)(6pter→6q15::4q21–q22::hsr::6q?::3q?27→3qter).

rangements in the cell lines and to identify all partner chromosomes involved in translocations with chromosome 4. The MCF-7 and S1 parent lines showed no rearrangements of chromosome 4 (as also shown with WCP 4). A single rearrangement of chromosome 4, a balanced reciprocal translocation, t(4;17)(q21–q22;p13), was seen in S1-M1–80 (Fig. 2a), while multiple translocations involving segments of chromosome 4 and a variety of partner chromosomes were seen in the other two sublines: MCF AdVp3000 had 16 different translocations, 8 of which were clonal (i.e., seen in two or more cells; Fig. 2b), and MCF MX had 11 translocations, 5 of which were clonal (Fig. 2c). These results are consistent with chromosomal instability accompanying gene amplification.

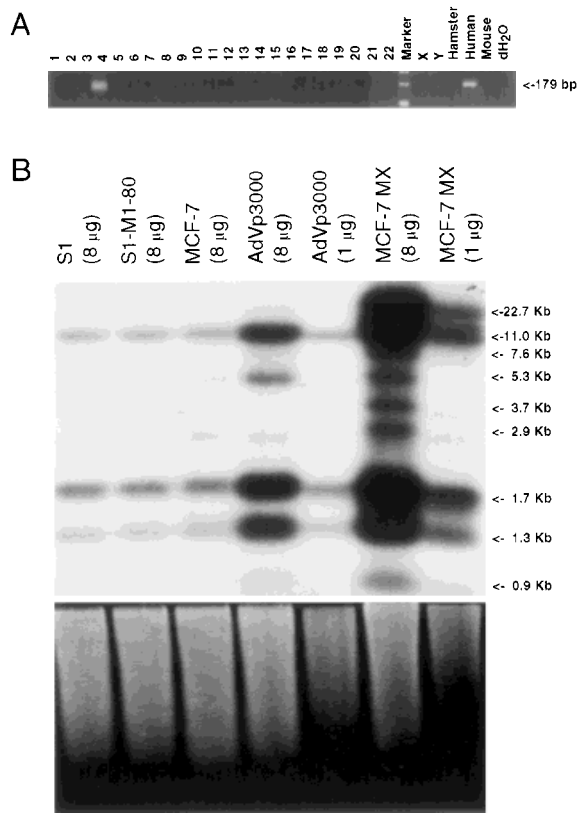

Figure 3. **A:** Localization of *MXR1* sequences to chromosome 4 by somatic cell hybridization. **B:** Southern blot analysis of the *MXR1* gene in the mitoxantrone-resistant sublines. DNA from the various cell lines was digested with *Eco*RI, purified, and separated on a 1% agarose gel. Lanes are as marked. The lanes from S1 parental, S1-M1–80, and MCF-7 parental cells contain 8-µg DNA, while those from MCF AdVp3000 and MCF MX cells contain either 8 µg or 1 µg. The blot was hybridized with random-primer labeled *MXR1* cDNA at 42°C in 50% formamide.

During the course of these studies, we isolated by differential screening two cDNAs from S1-M1–80 cells and demonstrated that both were overexpressed in the drug-resistant cell lines. We designated these *MXR*, for mitoxantrone-resistant (GenBank accession numbers *225118*, *225139*). Sequence analysis allowed for the design of primers which, as shown in Figure 3, localized these cDNAs to chromosome 4 using somatic cell hybrids (Coriell Cell Repositories, Mapping Panel *2*, version 3, Camden, NJ). A search of the EST database revealed that the two cDNAs differed primarily in length from human EST 157481, one of 21 ABC transporters previously reported by Allikmets et al. (1996) and localized to 4q22–q23. The full-length cDNA for EST 157481 was also cloned from placenta, sequenced, and termed *ABCP*, since high levels of expression were observed in placenta (Allikmets et al., 1998). The *MXR* cDNAs were thus likely candidates for the gene amplification observed in the CGH study, and Southern blot analysis was performed using random-primer-labeled *MXR* cDNA. Panel B of Figure 3 shows the results of a 16-hr exposure. Amplification of *MXR* is observed in the MCF-7 AdVp3000 cells (8–10-fold) and in the MCF-7 MX cells (20–24-fold), but not in S1-M1–80 cells, a finding that is consistent with the CGH results.

FISH analysis using an *MXR* BAC probe (> 50 kb and containing the 3′ end of the gene; BAC clone 346B24, Cambridge Institute of Technology BAC library, Research Genetics) was performed to localize the *MXR* gene to normal and rearranged chromosomes. The BAC hybridization procedure used is similar to other FISH technologies. The gene was localized to 4q21–*22* in normal lymphocytes, in both parental lines, and in the cytogenetically normal chromosomes 4 in the resistant lines (Fig. 4). In S1-M1–80 it was seen, without amplification, at the juncture of the t(4;17) translocation (Fig. 4). In retrospect, this translocation involving chromosome 4 had been seen previously using classical cytogenetic techniques, but its significance was unknown at that time (Rabindran et al., 1998). In MCF-7 AdVp3000 and MCF-7 MX, *MXR* was seen to be amplified at the juncture of one clonal translocation in each line, t(4;5) and t(6;4;6;3) (Fig. 4). In previous studies (Mickley et al., 1997; Knutsen et al., 1998), we demonstrated that random chromosome rearrangements, such as translocation and inversion, occurred in multidrug-resistant cell lines and samples from patients with refractory leukemia. Chromosomal rearrangements were also observed in the present study. Current efforts are directed at characterizing sequences juxtaposed to *MXR* to determine whether or not these sequences could confer the increased expression observed in S1-M1–80. Because MCF-7 AdVp3000 and MCF MX have amplification of *MXR* in the absence of amplification of genes that map to the partner chromosome translocations, it is less likely that the juxtaposed chromosome sequences have a significant role in the overexpression of *MXR*. However, future studies will be directed at determining whether early steps of resistance show translocations as an initial event prior to amplification (Mickley et al., 1997).

These data are consistent with the hypothesis of a common transporter, presumably that encoded by the *MXR* gene, mediating mitoxantrone resistance in these sublines. As currently understood, transporters able to extrude xenobiotics from the cell are members of the ABC (ATP binding cassette) superfamily of membrane transporters (Dean and Allik-

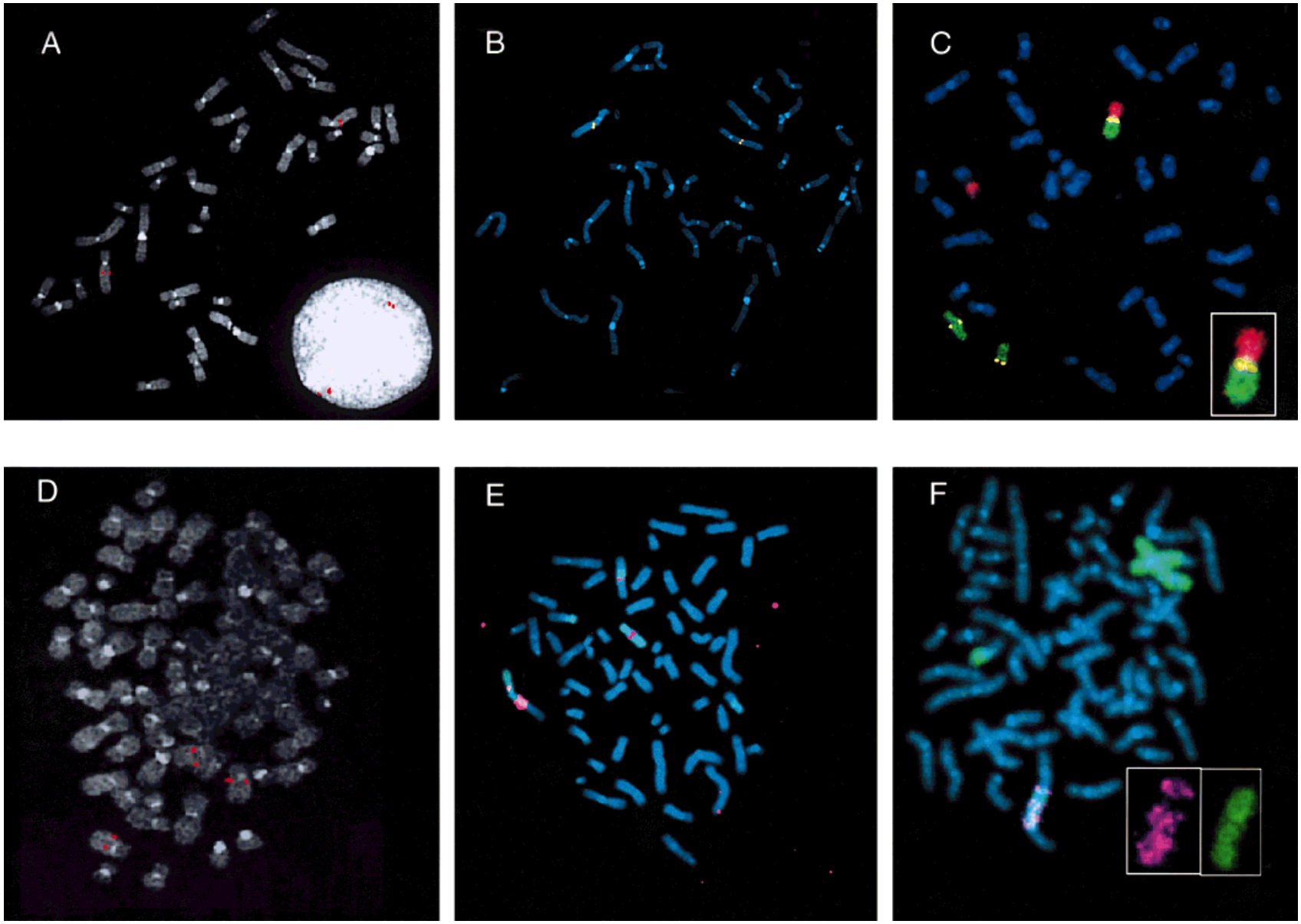

Figure 4. FISH with BAC-derived *MXR* probe localized to 4q21–22. Chromosome 4 is painted with WCP 4 spectrum green; the *MXR* probe was directly labeled with Cy5 or indirectly labeled with digoxigenin and detected with rhodamine anti-dig; DAPI counterstain. *MXR* is localized to 4q21–22 in (**A**) Normal lymphocytes: two normal chromosomes 4. **B:** S1 parent line: two normal chromosomes 4. **C:** S1-M1–80: one normal chromosome 4; t(4;17)(q21–q22;p13); the *MXR* signal is split at the breakpoint. **D:** MCF-7 parent line: three normal chromosomes 4. **E:** MCF AdVp3000: one normal chromosome 4, one add(4) with single *MXR* signal, and t(4;5) with amplified *MXR* signal at the translocation breakpoint. **F:** MCF MX: t(6;4;6;3) with amplified *MXR* signal at the translocation breakpoint.

mets, 1995). P-glycoprotein is the most well-known and widely studied, but the family also includes the multidrug resistance-associated protein, MRP, and the MRP homologue MRP2 (or cMOAT), as well as others (Borst et al., 1997). MRP mediates resistance to a variety of chemotherapeutic agents and can transport glutathione and glucuronide conjugates (Jeditsky et al., 1996). The MRP homologue, MRP2 (cMOAT), has as its normal role in human physiology the transport of bilirubin glucuronide and has been thought by some to confer resistance to cisplatin, although this has not been conclusively shown (Taniguchi et al., 1996; Koike et al., 1997).

The identification of *MXR* amplification and overexpression (Miyake et al., 1999) introduces half-transporters to the field of human cancer drug resistance. In addition to the cloning of *MXR* from SI-MI-80 and *ABCP* from placenta, Doyle et al. (1998) cloned an almost identical cDNA (termed *BCRP*) from adriamycin-resistant MCF-7 cells (MCF-7 AdVp). The *MXR/ABCP/BCRP* cDNAs demonstrate greatest homology with half-transporter genes such as the *Drosophila white* eye pigment and the *TAP* (Transporter Associated with Antigen Processing) genes, which encode half-transporter molecules that undergo heterodimerization to form a complete transporter. The Human Gene Nomenclature Committee (HUGO) recently recommended that *white* family genes be designated *ABCG* and *MXR/BCRP/ABCP* be designated *ABCG2* (Human ABC-Transporter Genes, www.gene.ucl.ac.uk/users/hester/abc.html).

Present efforts are directed at identifying potential partners for heterodimerization in the mitoxantrone-resistant sublines. The data in the MCF-7 sublines suggest that potential partners are either in close proximity to *MXR* or are not involved in drug resistance, because no other areas of amplification are shared by the cell lines. If an overexpressed partner cannot be found, it would suggest that at high levels of expression these half-transporters can homodimerize and function to transport drugs.

In summary, the present study demonstrates amplification of 4q21–q22 by CGH and cDNA hybridization, as well as rearrangements involving chromosome 4 by WCP 4 and SKY in three drug-resistant cell lines with high levels of mitoxantrone resistance. Together with evidence demonstrating amplification of a half-transporter designated *MXR*, which is located in this region, our findings suggest that chromosomal changes involving 4q21–q22 are important in this novel drug resistance phenotype. Numerous avenues remain to be explored, including the expression of the *MXR* gene in normal tissues, their potential importance in normal physiology, their significance in clinical drug resistance, and approaches to resistance reversal. In cytogenetic studies, chromosomal changes involving 4q21–q22 should be considered to have a potential role in drug resistance.

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