Activation of the MDRI Upstream Promoter in Breast Carcinoma as a Surrogate for Metastatic Invasion

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ABSTRACT

Purpose: Activation of the MDRI upstream promoter (USP) has been described previously in four lymphoblastic leukemia patients, where it is the major MDRI promoter associated with P-glycoprotein overexpression. We asked whether MDRI USP-derived transcripts were also present in breast carcinoma and assessed their potential as a biomarker.

Experimental Design: We developed a sensitive method for detecting transcripts derived from the MDRI USP and used it to identify MDRI USP-derived transcripts in cell model systems, in 61 breast carcinoma biopsies of the primary tumor, and in isolated malignant epithelial cells both from the primary tumor and from the associated invaded lymph nodes.

Results: The MDRI USP was not active in several independent leukemic and breast cancer cell lines or nucleated peripheral blood cells (n = 9). However, transcripts derived from the MDRI USP were detected in some drug-resistant cell lines and a high proportion of primary breast tumors (71.6%; n = 61), whereas they were present at low frequency in normal breast tissue (10%; n = 10). Activation of MDRI USP was not due to chromosomal amplifications or rearrangements at the MDRI locus. Transcription from the MDRI USP correlated with metastatic node invasion [N = 0–3 versus N > 3 (N = number of lymph nodes invaded); Fisher’s exact test, P = 0.011] and was detected in malignant epithelial cells from the primary tumor and those that metastasized to the lymph nodes.

Conclusions: MDRI USP activation is a surrogate marker for breast carcinoma progression and can be used as a marker to study breast cancer susceptibility.

INTRODUCTION

Despite advances in understanding the molecular causes of breast cancer, treatment of patients is still based primarily on the evaluation of a series of macroscopic and microscopic prognostic markers such as tumor size, grade of differentiation, histological type, and lymph node invasion (1). The only molecular marker widely used to predict treatment outcome in the clinic is estrogen receptor (ER) status (2, 3). Other markers are currently being evaluated in clinical studies (4–7), and progesterone receptor and HER2/neu receptor (8) are already used in some clinics. There is, therefore, a requirement for new molecular markers that could aid in guiding treatments, for example, to predict which tumors will eventually become invasive and metastatic, and which will remain localized and indolent (9–11). The use of highly sensitive detection methods, such as PCR-based technologies, may also facilitate the development of detection tools to detect micrometastatic disease in the follow-up of patients after mastectomy (12, 13).

The MDRI gene codes for P-glycoprotein, an ATP-dependent membrane transporter, which pumps many cytotoxic drugs out of the cells and hence, when overexpressed, can confer resistance to chemotherapy (14). Expression of P-glycoprotein in breast tumors is associated with poor response to chemotherapy (15). Two MDRI promoters were originally described in highly drug-resistant derivatives of human cervical carcinoma KB cell lines (16), the major downstream promoter [DSP (which is used by most cell lines and tissues expressing the MDRI gene)] and a minor upstream promoter [USP (which is not active in cell lines or healthy human tissues; Refs. 17–19)]. However, in acute lymphoblastic leukemic patients who overexpress P-glycoprotein, the MDRI USP represents the major promoter (and in some patients, the only promoter) used by mononuclear cells (20).

Here we show that the presence of transcripts derived from the MDRI USP in breast cancer tumors correlates with metastatic invasion of lymph nodes. We also detected activation of the MDRI USP in isolated carcinoma cells, both from the primary tumor and from invaded lymph nodes. Activation of the MDRI USP may then represent a marker associated with the metastatic phenotype.

MATERIALS AND METHODS

Patients. Seventy-one archival breast biopsies were used. Ten were from non-breast carcinoma patients (four normal tissue biopsies obtained after reductive mammoplasty and six from benign breast tumors). Sixty-one were from breast carcinoma patients. The mean age of the breast carcinoma patients was 56 years (range, 29–99 years); 25 of 37 patients had lymph...
node involvement; 2 patients had grade I disease, 20 patients had grade II disease, and 18 patients had grade III disease; and 23 of 31 patients were ER positive. Only one patient had received neoadjuvant chemotherapy with mitoantrone, methotrexate, and mitomycin C (21) before biopsy. Biopsies were collected at surgery and snap-frozen in liquid nitrogen.

Epithelial cells from five additional invasive ductal carcinoma patients and two reductive mammoplasty patients were purified, and purification was performed immediately after surgery. Three breast carcinoma patients had grade II disease, and two patients had grade III disease; four patients had N > 10 (N = number of lymph nodes invaded) and were ER and progesterone receptor negative, whereas one patient scored N = 3 and was positive for ER and progesterone receptor markers. These patients had not received neoadjuvant therapy.

Research into the MDR1 USP was approved by The Hammersmith Hospital Research Ethics Committee.

Purification of Epithelial Cells from Primary Tumors and Lymph Nodes. Carcinoma cells were immunofluorescence purified from normal breast tissue, primary tumors, and invaded lymph nodes after collagenase digestion using superparamagnetic polystyrene beads (Dynal, Wirral, United Kingdom) coated with mouse monoclonal antibody Ber-EP4, as described previously (22). The purity of the preparations was determined by immunohistochemistry for cytokeratins 8 and 18 (22). More than 98% of the purified cells were of epithelial origin.

Cell Lines. The vinblastine-selected, multidrug-resistant human cervical carcinoma mutant line KB-V1; leukemic cell line K562; and breast carcinoma cell lines MCF7, MDA-MB-231, MDA-MB-453, BT474, and CAL51; data for the multidrug-resistant derivatives MCF7AdrR and KD30 have been described previously (27).

RESULTS

Novel Splice Variants in Transcripts Derived from the MDR1 USP. Using a reverse transcription-PCR approach, we detected three transcripts derived from the MDR1 USP in KB-V1 cells (Fig. 1A). Sequence analysis indicated that the longer product (Fig. 1B, Transcript A) corresponded to the transcript originating from the USP, described previously (17). However, the other two products (Fig. 1B, Transcripts B and C) corresponded to previously unreported splice variants. In transcript B, an intron (Fig. 1B, IVS-1A) is spliced within exon 1, originating exons 1c and 1d, whereas in transcript C, a new intron (IVS-1b, comprising IVS-1a, exon 1d, and IVS-1) is spliced between exon 1c and exon 2.

Transcripts derived from the MDR1 USP were not detected in several leukemic or breast cancer-derived cell lines (K562, MCF7, MDA-MB-231, MDA-MB-453, BT474, or CAL51; data not shown), in agreement with previous reports (17). However, the MDR1 USP was active in MCF7AdrR, a cell line that, like KB-V1, overexpresses P-glycoprotein and is able to grow in the presence of doxorubicin-resistant derivatives MCF7AdrR and KD30 have been described previously (231, MDA-MB-453, BT474, and CAL51; their multidrug-resistant human cervical mutant line KB-V1; leukemic cell line K562; and breast carcinoma cell lines MCF7, MDA-MB-231, MDA-MB-453, BT474, or CAL51; data not shown), in agreement with previous reports (17). However, the MDR1 USP was active in MCF7AdrR, a cell line that, like KB-V1, overexpresses P-glycoprotein and is able to grow in the presence of doxorubicin-resistant derivatives MCF7AdrR and KD30 have been described previously (23).
presence of 1 μM doxorubicin (Fig. 2A). By successive selection with doxorubicin, we obtained K562 sublines able to grow in the presence of toxic concentrations of the drug ranging from 30 to 225 nm (this report and Ref. 27). Interestingly, the MDR1 USP was activated only in KD225 (Fig. 2A), whereas the other cell lines. MCF7AdrR showed essentially the same pattern of USP-positive cell line KB-V1 the MDR1 locus was activated only in KD225 (Fig. 2A). However, real-time reverse transcription-PCR showed up-regulation of total MDR1 mRNA, which detects transcripts derived from both the USPs and DSPs in all drug-resistant lines (Fig. 2A). Therefore, in these cell lines, as in KB-V1 (18), activation of MDR1 USP occurs after multiple rounds of selection for higher levels of resistance.

**Activation of MDR1 USP Is Not Due to Chromosomal Amplification or Rearrangements of the MDR1 Locus.** Because in the MDR1 USP-positive cell line KB-V1 the MDR1 locus is highly amplified (Ref. 32; Fig. 2C), we performed genomic Southern analysis to determine whether amplification or rearrangements of the MDR1 locus had occurred in the other cell lines. MCF7AdrR showed essentially the same pattern of amplification as KB-V1 cells (data not shown), with a MDR1:β-globin hybridization signal ratio suggesting that the locus was amplified 100-200-fold. A major and several minor chromosomal rearrangements had occurred in these lines such that the MDR1 probe hybridized to several DNA fragments in addition to the bona fide 6-kb genomic EcoRI fragment (Fig. 2B). In contrast, in the K562 derivative KD225, activation of MDR1 USP occurred without detectable amplification or rearrangement of the locus (Fig. 2C). Therefore, activation of MDR1 USP can occur without gross genomic abnormalities in the locus, and its transcripts must then be the result of transcriptional activation.

**Activation of the MDR1 USP in Primary Breast Tumors Correlates with Metastatic Spread.** MDR1 USP usage was found in 44 of 61 breast carcinoma biopsies (Fig. 3A). Only one breast tissue sample originated from a patient who had received adjuvant chemotherapy (which scored USP positive), and, therefore, the percentage of MDR1 USP usage at the time of surgery was high (71.6%). In contrast, only 1 of the 10 non-malignant breast tissue samples showed MDR1 USP activation (Fig. 3A). This sample was from a reductive mammoplasty and did not show any histological abnormalities. Transcript A was the most abundant in all positive samples, whereas transcript C was absent in some patients (Fig. 3B). Total MDR1 mRNA, detected by amplification of exons 6-8, was present in most samples and was probably due to lymphocyte infiltration (because lymphocyte marker CD2 was also expressed in most samples) or to small amounts of blood in the sample (data not shown). It is well established that several subpopulations of lymphocytes express MDR1 mRNA to different extents (33). We have not detected activation of the MDR1 USP in lymphocytes from healthy volunteers (n = 9), thus all of the MDR1 transcripts in these cells originate from the DSP. The fact that lymphocytes are present in most tissue samples obtained from patients highlights the technical problems in determining the MDR1 expression status of tissues by a highly sensitive technique such as PCR, which does not distinguish between tissue-derived and lymphocyte-derived transcripts.

We performed genomic Southern analysis to ascertain whether amplification of the MDR1 locus had occurred in the MDR1 USP-positive tumors, as observed for highly drug-resis-
tant cell lines. Four USP-positive and four USP-negative samples were analyzed, and the \( \text{MDR1}^{\text{USP}} / \text{globin} \) hybridization signal ratio was the same in the two groups (Fig. 3C), showing that, in breast carcinoma patients, activation of the \( \text{MDR1}^{\text{USP}} \) is not a consequence of DNA amplification.

There was no significant correlation between \( \text{MDR1}^{\text{USP}} \) activation and patient age (\( \text{P} = 0.58 \)), tumor size (\( \text{P} = 0.94 \)), grade of differentiation (\( \text{P} = 0.66 \)), and ER status (\( \text{P} = 0.19 \)). However, \( \text{MDR1}^{\text{USP}} \) usage in the primary tumor did correlate with the number of lymph nodes with metastatic cells. All \( n = 10 \) patients with four or more lymph nodes with metastasis showed activation of the \( \text{MDR1}^{\text{USP}} \) (Fig. 4A). Fisher’s exact test indicated a difference (\( \text{P} = 0.011 \)) between this group of patients and those who had none or one to three nodes invaded. Interestingly, patients with less than four nodes invaded showed activation of \( \text{MDR1}^{\text{USP}} \) in approximately 50% of cases.

**Malignant Metastatic Breast Epithelial Cells Have an Activated MDR1 USP.** To ascertain whether carcinoma cells showed activation of the \( \text{MDR1}^{\text{USP}} \), we purified them from fresh tumor biopsies by binding to a monoclonal antibody against the Ber-EP4 epithelial marker (34). Five patients with 3–23 invaded lymph nodes were selected, and all scored positive for \( \text{MDR1}^{\text{USP}} \) activation (Fig. 4B). Because of the strong correlation between \( \text{MDR1}^{\text{USP}} \) activation and lymph node invasion, we asked whether the metastatic cells present in the lymph nodes from two of the above patients had an activated \( \text{MDR1}^{\text{USP}} \). Both were positive (Fig. 4B). Interestingly, we detected activation of the \( \text{MDR1}^{\text{USP}} \) in one of two control samples derived from reductive mammoplasty.

**DISCUSSION**

Activation of the \( \text{MDR1}^{\text{USP}} \), first described in a highly drug-resistant cell line (17, 18), has been detected previously in 4 of 28 patients with acute lymphoblastic leukemia (20), where it becomes the major and sometimes sole active \( \text{MDR1}^{\text{USP}} \) promoter. In this study we demonstrate the presence of transcripts, some of which are novel splice variants, derived from the \( \text{MDR1}^{\text{USP}} \) in a high proportion (71.6%) of primary tumors from breast carcinoma patients. Changes in global gene expression in breast...
cancer have been reported using microarray technology (10, 11, 35), and overexpression of MDR1 has been associated with the ER-negative/HER2-neu-positive subgroup (36). However, the probes used to detect MDR1 mRNA in these array experiments do not differentiate between the transcripts derived from either the USP or the DSP, making it difficult to compare our data. Although MDR1 locus amplification is a common event in cell lines (23, 37), it has never been specifically described in human neoplasms (38, 39). However, amplification of 7q21, the chromosomal region where the MDR1 locus lies, has been described in one head and neck squamous cell carcinoma (40) and four malignant glioma (41) cases. In addition, random chromosomal rearrangements between MDR1 and other sequences have been described in two patients with drug-refractory acute lymphoblastic leukemia (42). In this study, we have eliminated MDR1 USP activation as a consequence of gene amplification. Southern analysis of both cell lines and patient biopsies indicated that amplification of the MDR1 locus had taken place (Figs. 2B and 3). However, we cannot rule out the possibility that breast cancer patients with activation of the MDR1 USP have microdeletions or that the percentage of tumor cells containing amplifications was so low as to be beyond the detection limits of the techniques used.

Because no transcripts derived from the MDR1 USP are found in cell lines and most nonmalignant breast tissue samples, we can conclude that this promoter is located in a normally silent region of the genome. Yet in cells exposed to high doxorubicin concentrations, like KD225 and MCF7AdrR, the normally silent MDR1 UP is activated (Fig. 2A). Although many drugs produce DNA hypermethylation, at the concentrations used here doxorubicin induces DNA hypomethylation (43, 44), a phenomenon associated with gene activation (45, 46). Our data indicate that the MDR1 USP region becomes transcriptionally competent in a high proportion of breast cancer patients. All patients examined with a high number of invaded nodes (N > 3; n = 10) scored positive for the MDR1 USP in the primary tumor, whereas in patients with N < 3, the marker was present in 52% of cases (14 of 27 cases); these facts indicate that activation of MDR1 USP is associated with tumor progression and metastatic spread. One of the characteristics of breast cancer development is a progressive global DNA hypomethylation, and the DNA methylation status of a tumor has been suggested as a biological marker with prognostic significance (47, 48). Transcriptional activation of MDR1 by hypomethylation of the DSP has been demonstrated in acute myeloid leukemia (49). However, the MDR1 DSP is located in a CpG island, a region of the genome rich in the dinucleotide CpG and associated with many gene promoters (45, 46), whereas the MDR1 USP is not. Therefore, although the progressive global demethylation occurring in
breast cancer might have a role in activating the \(\text{MDR1}\) USP, we cannot rule out the possibility of activation of transcription factors specific to the metastatic phenotype (50). Although we do not know the proportion of \(\text{MDR1}\) transcripts originating from the DSP and USP in breast cancer patients before chemotherapy, in acute lymphoblastic leukemic patients, who overexpress P-glycoprotein after chemotherapy, the \(\text{MDR1}\) USP represents the major promoter used by mononuclear cells (20). In a cell expressing \(\text{MDR1}\) mRNA from the DSP, the presence or absence of transcripts derived from the USP would not notably change its P-glycoprotein status because the start codon present in exon 2 is not affected in either of the splice variants or the transcript originating from the DSP (Fig. 1B). Although the role of P-glycoprotein in response to chemotherapy in breast cancer is still debated (51), many studies indicate that patients expressing P-glycoprotein are more likely to fail to respond to chemotherapy than patients whose tumors are P-glycoprotein negative (15, 52). Moreover, in MCF7AdR cells, drugs transported by P-glycoprotein induce membrane ruffling, which is an early indicator of cellular motility and metastatic potential (53). Therefore, the possibility that some chemotherapeutic agents may promote metastasis cannot be ruled out. However, no correlation between P-glycoprotein expression and lymph node metastasis before chemotherapy has been found previously (15). We favor the interpretation that the association between \(\text{MDR1}\) USP activation and metastatic lymph node invasion reported here represents a surrogate to mark other biological processes such as global demethylation or activation of transcription factors associated with the metastatic phenotype and acting on the \(\text{MDR1}\) USP. Expression of both P-glycoprotein and mutant p53 has been reported to be associated with poor prognosis of breast cancer (54), which could also suggest that the \(\text{MDR1}\) USP activation is linked to the expression of a gene or genes involved in tumor progression and metastasis. Which of these mechanisms is correct will require additional studies on the \(\text{MDR1}\) USP.

Although we have analyzed a relatively small sample of normal breast tissues for population studies, the results shown here would suggest a relatively low frequency for \(\text{MDR1}\) USP activation. In the group of breast carcinoma patients analyzed, frequency significantly increases to 71.6% and becomes 100% in those with a high number of metastasized lymph nodes. Therefore, we can conclude that activation of the \(\text{MDR1}\) USP can be used as a surrogate marker for breast cancer progression and metastatic spread, opening the possibility of using it to study breast cancer susceptibility.

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REFERENCES

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