Activation of the \textit{MDR1} Upstream Promoter in Breast Carcinoma as a Surrogate for Metastatic Invasion

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\section*{ABSTRACT}

\textbf{Purpose:} Activation of the \textit{MDR1} upstream promoter (USP) has been described previously in four lymphoblastic leukemia patients, where it is the major \textit{MDR1} promoter associated with P-glycoprotein overexpression. We asked whether \textit{MDR1} USP-derived transcripts were also present in breast carcinoma and assessed their potential as a biomarker.

\textbf{Experimental Design:} We developed a sensitive method for detecting transcripts derived from the \textit{MDR1} USP and used it to identify \textit{MDR1} 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.

\textbf{Results:} The \textit{MDR1} USP was not active in several independent leukemia and breast cancer cell lines or nucleated peripheral blood cells (\textit{n} = 9). However, transcripts derived from the \textit{MDR1} USP were detected in some drug-resistant cell lines and a high proportion of primary breast tumors (71.6\%; \textit{n} = 61), whereas they were present at low frequency in normal breast tissue (10\%; \textit{n} = 10). Activation of \textit{MDR1} USP was not due to chromosomal amplifications or rearrangements at the \textit{MDR1} locus. Transcription from the \textit{MDR1} USP correlated with metastatic node invasion [\textit{N} = 0–3 versus \textit{N} > 3 (\textit{N} = number of lymph nodes invaded); Fisher’s exact test, \textit{P} = 0.011] and was detected in malignant epithelial cells from the primary tumor and those that metastasized to the lymph nodes.

\section*{INTRODUCTION}

\textbf{Purpose:} The \textit{MDR1} USP activation is a surrogate marker for breast carcinoma progression and can be used as a marker to study breast cancer susceptibility.

\section*{MATERIALS AND METHODS}

\textbf{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 mitoxantrone, 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 immunoaffinity 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; leukemia cell line K562; and breast carcinoma cell lines MCF7, MDA-MB-231, MDA-MB-453, BT474, and CAL51 and their multidrug-resistant derivatives MCF7AdrR and KD30 have been described previously (27). Carcinoma cells were isolated from cells and some biopsies (both from \( MDR1 \) USP-positive and -negative patients) by proteinase K digestion and phenol/chloroform extraction, digested with \( EcoRI \), Southern blotted, and hybridized to nick-translated radioactive probes by standard procedures (31). \( MDR1 \) and \( \beta \)-globin loci probes have been described previously (27). \( MDR1: \beta \)-globin ratios were obtained by quantitation using a PhosphorImager.

**Statistical Analysis.** Because the continuous data did not follow normal distributions, a nonparametric approach was used (Mann-Whitney \( U \) test). For tabular data, Fisher’s exact test was used. Statistical significance of each test was assumed when \( P < 0.05 \).

**RESULTS**

**Novel Splice Variants in Transcripts Derived from the \( MDR1 \) USP.** By 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, \( I V S-1a \)) 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

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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 neoadjuvant 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 nonmalignant 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 MDR1 USP activation and patient age \( (P = 0.58) \), tumor size \( (P = 0.94) \), grade of differentiation \( (P = 0.66) \), and ER status \( (P = 0.19) \). However, MDR1 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 MDR1 USP (Fig. 4A). Fisher’s exact test indicated a difference \( (P = 0.011) \) 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 MDR1 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 MDR1 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 MDR1 USP activation (Fig. 4B). Because of the strong correlation between MDR1 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 MDR1 USP. Both were positive (Fig. 4B). Interestingly, we detected activation of the MDR1 USP in one of two control samples derived from reductive mammoplasty.

**DISCUSSION**

Activation of the MDR1 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 MDR1 promoter. In this study we demonstrate the presence of transcripts, some of which are novel splice variants, derived from the MDR1 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 all cell lines and patient biopsies indicated that MDR1 USP-negative scores, Fisher’s exact test indicated that the activation of the MDR1 USP in breast carcinoma was statistically significant ($P = 0.0006$) when compared with normal tissue. B, reverse transcription-PCR detection of transcripts derived from the MDR1 USP. Sixty-one patient biopsies were analyzed; only 10 are shown for clarity. All 10 normal breast tissue samples analyzed are shown. A–C correspond to the different splice variants described in Fig. 1. Transcript A was the most abundant, and not all USP-positive samples showed the least abundant transcript, transcript C. C, activation of MDR1 USP in breast carcinoma patients is not a consequence of gene amplification. Southern blot analysis (as in Fig. 2B) of eight biopsies from breast carcinoma patients (four were positive for MDR1 USP activation, and four were negative for MDR1 USP activation) is shown. MDR1 USP isoforms were shown to be negative between MDR1 USP-positive and normal patients.

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 USP is activated (Fig. 2A). Although many drugs produce DNA hypermethylation, at the concentration 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 \textit{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 \textit{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 \textit{MDR1} USP represents the major promoter used by mononuclear cells (20). In a cell expressing \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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**


![Fig. 4 MDR1 upstream promoter (USP) activation and breast cancer metastasis. A, correlation with lymph node invasion. Breast carcinoma patients were distributed into three groups according to the degree to lymph node metastatic invasion and the score for the \textit{MDR1} USP status plotted (negative, ●; positive, ■). All patients (n = 10) with a high number of lymph nodes invaded (poor prognostic indicator) showed activation of the \textit{MDR1} USP. Fisher’s exact test indicated a statistical difference between the N > 3 and N = 0–3 groups (N = number of lymph nodes invaded; P = 0.011). B, activation of the \textit{MDR1} USP in isolated epithelial cells. Cells were isolated by immunoaffinity to the epithelial marker Ber-EP4 from primary tumors from five patients (P1–P5) showing lymph node invasion, from the lymph nodes of two patients (P4 and P5), and from two normal breast tissue biopsies from reductive mammoplasty. Detection of \textit{MDR1} USP-derived transcripts A–C was performed as described in Fig. 1A.](image-url)

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