

Research article

A candidate metastasis-associated DNA marker for ductal mammary carcinoma

Patnala Mohan R Achary¹, Hui Zhao¹, Zuoheng Fan¹, Swarna Gogineni², Venkat R Pulijaal², Lawrence Herbst³, Panna S Mahadevia³, Joan G Jones³, Harold P Klinger⁴ and Bhadrasain Vikram¹

¹Metastasis Laboratory, Department of Radiation Oncology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York, USA

²Department of Obstetrics and Gynecology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York, USA

³Department of Pathology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York, USA

⁴Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York, USA

Corresponding author: Patnala Mohan R Achary (e-mail: achary@aecom.yu.edu)

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Abstract

Background: Molecular genetic markers to identify the 13% lymph node-negative mammary carcinomas that are prone to develop metastases would clearly be of considerable value in indicating those cases in need of early aggressive therapy.

Methods: Representational difference analysis was used in an attempt to identify genetic alterations related to breast cancer metastasis by comparing genomic DNA from microdissected normal cells and from metastatic cells of ductal breast carcinoma patients.

Results: Representational difference analysis products yielded 10 unique metastasis-associated DNA sequences (MADS), i.e. products apparently lost in metastatic cell DNA. Of these sequences, MADS-IX was found to be lost in the transition from primary to metastasis in two out of five ductal breast

carcinoma cases. This sequence was localized on chromosome 10q21 by radiation hybrid mapping and fluorescence *in situ* hybridization. The *PTEN* gene, which is also located on chromosome 10q, was detected to be present by PCR in all five cases. On the contrary, a breast carcinoma cell line, HCC-1937, which has homozygous loss of a region encompassing the *PTEN* gene, showed the presence of MADS-IX. PCR screening of three additional breast carcinoma cell lines with known losses in specific chromosomal regions also showed the presence of MADS-IX.

Conclusion: These data suggest that MADS-IX possibly is part of a novel candidate metastasis-associated gene located close to the *PTEN* gene on chromosome 10q. The first set of PCR screening in five patient samples indicates that it could be used as a molecular marker for ductal mammary metastasis.

Keywords: archival tumor samples, mammary carcinoma, metastasis, molecular markers, representational difference analysis

Introduction

The clinical outcome is generally positive for patients with node-negative breast carcinoma (i.e. those patients without detectable metastases in the lymph nodes) who have been treated with surgery or with surgery and radiation therapy. The disease spreads in about 13% of the patients, however, and their lives are at risk of death [1–3]. Genetic tests capable of identifying patients at risk for

metastatic spread and/or better treatment targeted to eradicate metastatic tumor deposits could have a dramatic impact on the overall survival of these patients.

The construction of a panel of molecular genetic markers for identifying the 13% lymph node-negative mammary carcinomas that are prone to develop metastases [3] would be valuable in indicating those cases in need of early

bp = base pairs; FISH = fluorescence *in situ* hybridization; LCM = laser capture microdissection; MADS = metastasis-associated DNA sequences; NCBI = National Center for Biotechnology Information; PCR = polymerase chain reaction; RDA = representational difference analysis; SCM = single cell microdissection; STS = sequence tagged sites.

aggressive therapy. There would also be considerable benefit to the 87% of women with mammary carcinomas who are not likely to metastasize by sparing them the physical, mental and financial costs of the treatment. In addition, further knowledge of the genetic mechanisms that play an important role in metastasis could ultimately lead to the development of improved therapeutic procedures.

The evidence for the role of gene alterations in promoting metastasis in general, and in mammary carcinomas in particular, is still accumulating. It is probable, however, that like tumorigenicity suppressor genes and oncogenes many more genes involved in metastasis remain to be discovered since the process is complex, involving a large number of pathways [4]. Most of the known genetic mechanisms involved in the progression of a tumor to the metastatic state involve the loss of function of genes that prevent cells from becoming invasive. These are similar to tumorigenicity suppressor genes, whose loss of wild-type growth regulatory function leads to unregulated or malignant growth. Similarly, the wild-type metastasis suppressor genes regulate the cell's mobility and its response to environmental messages, presumably keeping most cells localized and differentiated. Mutation or complete loss (deletion) of the wild-type counterpart of any such gene in a malignant cell may make that cell more motile, invasive or metastatic. In particular, mutations of genes regulating cell adhesion molecules have been reported to be metastasis-fostering alterations [5–8]. In human breast cancers, several other genes have been found to be associated with metastasis, namely *nm23* [9,10], *KAI1* [11,12], *mta1* [13], *KiSS1* [14], *PTEN/MMAC1* [15,16] and *BRMS1* [17].

Representational difference analysis (RDA), a DNA subtractive hybridization method [18], was used in the present study to test the working hypothesis that, to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic changes in addition to those changes that led to transformation. With the RDA method, DNA from normal cells was compared with that of the metastatic cells of the same patient. The recovered differential sequences were then mapped on human chromosomes and used to screen DNA samples from normal, primary and metastatic cells of five additional ductal mammary carcinoma patients to determine whether these sequences were consistently associated with metastasis.

Materials and methods

Tissue samples and cell lines

Normal breast tissue and lymph nodes with metastatic cells of a 52-year-old patient (C-1050) with ductal mammary carcinoma were provided by the Cooperative Human Tissue Network. Additional matched normal, primary and metastatic tissue samples were collected from four patients (patient 1, C-18805; patient 2, C-98-05H; patient 3, C-19898; and patient 4, C-20635)

from the Cooperative Human Tissue Network and from one patient (patient 5, DS-9605) from the Surgical Pathology Department of Albert Einstein College of Medicine and Montefiore Medical Center. The DNA samples from mammary carcinoma cell lines (HCC-1806, HCC-11433, HCC-1428 and HCC-1937) and matched normal cell lines were received from Dr R Parson of Columbia University, New York, USA.

Isolation of cells from biopsy samples by single cell microdissection

Single cell microdissection (SCM) was performed on hematoxylin and eosin-stained tissue sections of positive lymph nodes from the ductal breast cancer patient samples [19]. In the present method of SCM, a Zeiss axiovert phase contrast photomicroscope fitted with a television monitor and a Narashige mechanical microdissector was used. A glass micropipette was used to draw up individual cells after dissection.

Tumor cells were identified in the microscope and, using the tip of a glass micropipette attached to a syringe, they were dissected out without disturbing the surrounding tissue and were then drawn into the pipette. After five to 10 cells were collected in the tip, it was broken off and dropped into a sterile eppendorf tube. Approximately 10,000 metastatic tumor cells were collected in this manner for RDA experiments. The SCM method was used to isolate tumor cells for the RDA experiment because laser-capture microdissection (LCM) equipment was initially not available.

Isolation of cells from biopsy samples by LCM

LCM [20] was used for isolating tumor cells from primary and metastatic tissue samples of additional patients for screening candidate metastasis-associated DNA sequences (MADS) by PCR. After many tumor cells have been captured on the LCM cap, it is then placed on a 500 μ l PCR tube containing ATL lysis buffer (Qiagen Inc, Valencia, CA, USA) and the DNA is extracted.

DNA extraction

About 1 μ g DNA was isolated from approximately 10,000 cells microdissected from metastatic tumor and normal (glandular and stromal) breast tissue samples, using a modified method of DNA extraction from archival tissues. Briefly, the microdissected cell pellet was incubated overnight in lysis buffer (Qiagen Inc) with proteinase-K at 55°C. Glycogen (carrier) was added to the cell lysate and DNA was extracted using a phenol–chloroform–isoamyl alcohol mixture followed by ethanol precipitation [19].

RDA and characterization of differential products

The RDA procedure was basically as described by Lisitsyn and coworkers [18,21] using the *Bgl*II representation. The metastatic cancer cell DNA was used as the 'driver'

and the DNA from the normal cells was the 'tester'. Three rounds of hybridization were performed to subtract the common sequences. The primers used for representation and hybridization steps of RDA are presented in Table 1. DNA from the differential bands (lost in metastasis) was cloned using the TA cloning system (pCR2.1; Invitrogen Co, Carlsbad, CA, USA). Clones were selected at random and used as $\alpha^{32}\text{P}$ -dCTP (Amersham Biosciences Corp, Piscataway, NY, USA) probes on dot blots or Southern blots containing amplicon DNA from the original normal and metastatic cells to verify whether these sequences are actually present in the normal cell DNA sample and missing in the metastatic cell DNA samples. Positive clones (showing signals in normal cell DNA and missing in metastatic cell DNA) were sequenced. A homology search was performed using the National Center for Biotechnology Information, National Institutes for Health, USA (NCBI) Blast program and physical mapping.

Radiation hybrid mapping

Radiation hybrid mapping was used to localize the MADS on human chromosomes using high-resolution GeneBridge 4 radiation hybrid panels (Research Genetics, Inc, Carlsbad, CA, USA). Based on the sequences of differential products isolated from RDA experiments, 10 pairs of PCR primers were designed for 10 groups of candidate gene sequences. Based on the PCR results, chromosome localization of these sequences was determined using the Whitehead Institute/MIT databases [22].

Fluorescence *in situ* hybridization of MADS-IX

Chromosome preparations were made from phytohemagglutinin-stimulated peripheral blood lymphocyte cultures from normal individuals. The slides were fixed in methanol-acetic acid and were air-dried. The probes used for fluorescence *in situ* hybridization (FISH) were the Spectrum Green labeled centromere of 8 (Vysis, Downers Grove, IL, USA) and the Spectrum Green labeled 2 kb sized MADS-IX sequence. Primers were designed from the BAC clone (NCBI, NIH, USA, RP11-407) containing the MADS-IX sequence to obtain a 2 kb sized PCR product encompassing the MADS-IX region (Table 1).

The 2 kb DNA fragment was run on a 1% gel, isolated from gel and eluted in water, and was labeled with Spectrum Green using the nick translation method, following the manufacturer's instructions (Vysis). Three-day-old slides were denatured in 70% formamide/2×SSC (sodium citrate/chloride) solution at 74°C. The dehydrated and air-dried slides were used for hybridization according to published methods with some modifications [23,24]. The slides were counter-stained with 4,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes, Eugene, OR, USA) and images were captured on the Nikon E800 using the Quips Pathvysion system (Applied Imaging, Santa Clara, CA, USA).

Results

Isolation of candidate MADS

RDA was performed with the DNA recovered by SCM from normal cells and from metastatic cells in the lymph nodes of a patient with ductal mammary carcinoma. Since the objective was to isolate sequences that are lost in the progression of cells from normal to metastasis, we focused on the products of RDA in which the metastatic cell DNA was used as the driver and the normal cell DNA was the tester.

As shown in Fig. 1, RDA differential products were isolated and DNA was extracted from each of the five bands obtained from the loss side of the third round of hybridization, and DNA from each band was cloned separately. One hundred clones from each DNA band were saved and a subset of 100 clones (20 clones from each differential product) was selected randomly for further characterization. These 100 clones were probed (labeled with $\alpha^{32}\text{P}$ -dCTP) on dot blots containing normal cell DNA and metastatic cell DNA. It was found that 79 clones hybridized only with the normal cell DNA and did not hybridize with DNA from the metastatic cells (Fig. 2a). The remaining clones hybridized with both normal cell DNA and the metastatic cell DNA. This may have been due to incomplete subtraction. Of the 79 clones that hybridized only with the DNA of the normal cells, 50 were selected and tested further by Southern blotting to verify the RDA results. None of these clones hybridized with the DNA of the metastatic cells, and all were detected in normal cells (Fig. 2b).

Sequencing and homology search of candidate MADS

RDA derived 50 clones that were sequenced and those with identical sequences were grouped. Ten unique candidate MADS were thus identified. The sequence homologies found by searching the nucleotide databases of these MADS are presented in Table 2. The search revealed that these MADS have homology with sequences on chromosome 1 (MADS-III), chromosome 5 (MADS-I), chromosome 6 (MADS-VII and MADS-VIII), chromosome 7 (MADS-VI), chromosome 10 (MADS-IX), chromosome 15 (MADS-X), chromosome 16 (MADS-V) and chromosome 21 (MADS-II). Nine of these MADS have about 82–99% homology to known human DNA sequences, and the remaining MADS-IV was found to be novel, having no homology with the sequences in the gene banks.

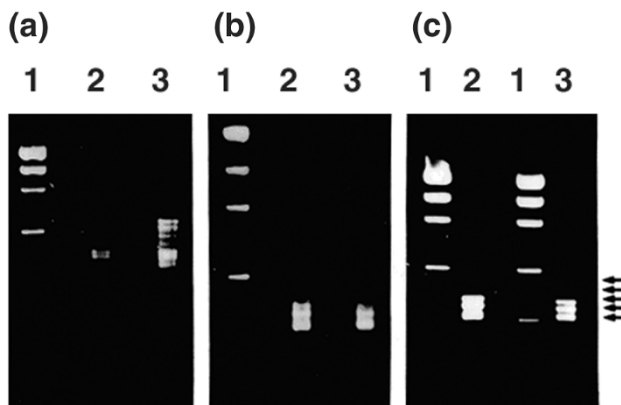
Radiation hybrid mapping of MADS

We used the GeneBridge 4 Radiation Hybrid panel (Research Genetics, Inc) to localize these 10 MADS on human chromosomes. For this physical mapping, and also to be able to use these sequences to screen additional patient samples, primer pairs were designed for each sequence and tested by PCR on a positive control (human genomic DNA) and on two negative controls (Chinese hamster genomic DNA and no template). Of the 10 MADS

Table 1**Primers used in representational difference analysis (RDA) and different PCR screening experiments**

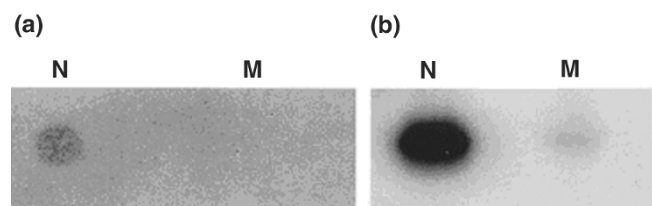
Primer/product size	Primer sequence (forward)	Primer sequence (reverse)
RDA representation	R24, 5'-agcactctccagcctctcaccgca-3'	R12, 5'-gatctgcggtga-3'
RDA first-round hybridization	J24, 5'-accgacgtcgactatccatgaaca-3'	J12, 5'-gatctgttcag-3'
RDA second-round hybridization	N24, 5'-aggcaactgtgctatccgaggaa-3'	N12, 5'-gatcttccctcg-3'
RDA third-round hybridization	Same as first round (J24)	Same as first round (J12)
<i>PTEN</i> (556 bp)	5'-ctcagattgccttataatagtc-3'	5'-tcattgtactgctacgtaaac-3'
MADS-IX (180 bp)	5'-aggtaggtagagtaacaggtttgtt-3'	5'-gatctgttccctttcttagctt-3'
FISH fragment (2 kb)	5'-actatgtatgtgacatctgtttgc-3'	5'-tgactcttactgagtaaactg-3'
β -Actin (295 bp)	5'-tcaccacactgtgcccactctacga-3'	5'-cgacgtacacagcttctccta-3'

FISH, fluorescence *in situ* hybridization.

Figure 1

Isolation of representational difference analysis (RDA) differential products from single-cell microdissected archival breast tissues. RDA hybridization was of the normal cell DNA versus the metastatic tumor cell DNA and was performed in two ways. In the first RDA (lane 2) the metastatic cell DNA was used as the tester (which should yield differential sequences gained during malignant transformation or in the process of becoming metastatic), and in the second RDA (lane 3) normal DNA was used as the tester (which should yield sequences that were lost from the metastatic cells). (a), (b) and (c) Three rounds of RDA hybridizations were performed. In the third round of hybridization, the 'gain' lane contained four DNA bands (faint fourth band) ranging from 200 to 300 bp in size (c, lane 2) whereas in the 'loss' lane there are five bands (faint fourth band) ranging from 200 to 370 bp (c, lane 3) in size (arrows). Lane 1, low molecular weight DNA marker (2 kb).

tested, only five (MADS-IV, MADS-VII, MADS-VIII, MADS-IX and MADS-X) were successfully mapped to specific human chromosomes (Table 2). Of these five MADS, MADS-IV did not show any match with gene bank sequences and therefore appeared to be a novel DNA sequence. The chromosome localizations for MADS-VII, MADS-VIII, MADS-IX and MADS-X obtained by physical mapping and by homology search data were in agreement.

Figure 2

Southern blot analysis using MADS-IX as an $\alpha^{32}\text{P}$ -dCTP probe on normal amplicon DNA and on metastatic amplicon DNA. (a) A dot blot and (b) a Southern blot showing positive signals only in the normal cell DNA (N) but not in the metastatic cell DNA (M).

Further characterization of MADS-IX

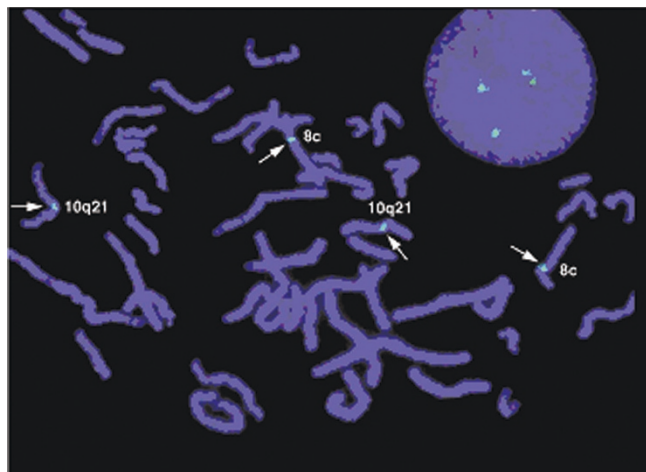
While the homology results of MADS-IX revealed that it has 94% homology with the sequence on chromosome 10q (BAC-AC022541.10), the radiation hybrid mapping showed that MADS-IX is localized to a 21cR interval between markers D105539 and D10S549, corresponding to human chromosome 10 band q21.1 [25]. To confirm that MADS-IX is not an artifact and is actually a human DNA sequence located on chromosome 10q, we used the sequence as a fluorescence *in situ* hybridization probe on human metaphase chromosomes. Based on the sequence of the BAC clone (NCBI, RP11-407) encompassing MADS-IX, a 2 kb DNA fragment containing MADS-IX was generated by PCR. To localize MADS-IX cytologically, we labeled the 2 kb sized MADS-IX with Spectrum Green (Vysis) and hybridized on normal human metaphase chromosomes. Figure 3 shows that the MADS-IX probe was hybridized on the 10q21 region. The Spectrum Green labeled centromere of chromosome 8 (Vysis) was used as a control.

To determine whether MADS-IX is indeed associated with metastasis, DNA was screened from cells recovered with the LCM method from normal, primary and metastatic tissue

Table 2

The length, homology search and radiation hybrid mapping results of the metastasis-associated DNA sequences (MADS)			
MADS	Length (bp)	Homology	Radiation hybrid mapping
MADS-I	205	Chromosome 5 (97%) AC005915.1	Not successful
MADS-II	144	Chromosome 21 (99%) AC010463.6	Not successful
MADS-III	277	Chromosome 1 (97%) AL359265.8	Not successful
MADS-IV	185	No match	Chromosome 5; places 19.72 cR from WI-6737
MADS-V	220	Chromosome 16 (98%) AC004234.1	Not successful
MADS-VI	242	Chromosome 7 (97%) AC09333.1	Not successful
MADS-VII	190	Chromosome 6 (98%) AL121935.17	Chromosome 6; places 1.51 cR from WI-3110
MADS-VIII	170	Chromosome 6 (82%) AC013429.12	Chromosome 6; places 3.67 cR from AFMA191WD1
MADS-IX	180	Chromosome 10 (94%) AC022541.10	Chromosome 10; places 5.66 cR from D10S546
MADS-X	192	Chromosome 15 (98%) AC104260.5	Chromosome 15; places 1.71 cR from D15S157

Figure 3

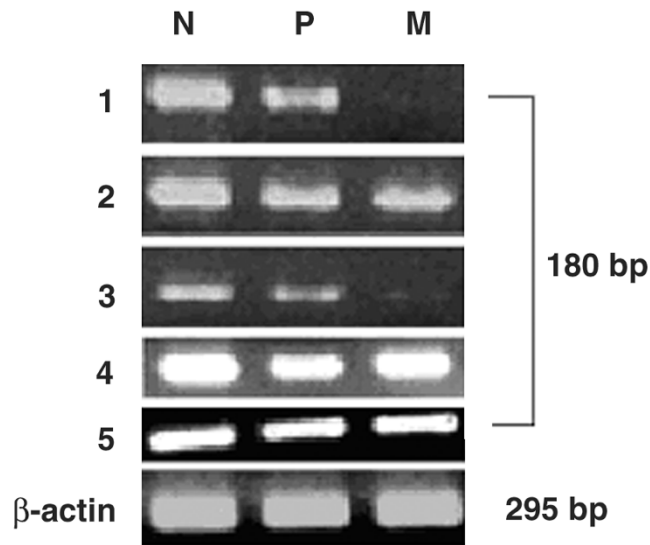


Localization of the 2 kb DNA fragment containing MADS-IX on human metaphase chromosomes. The centromere of chromosome 8 was used as a positive control probe. MADS-IX was found to localize around the 10q21 chromosome region (close to the centromere). Both the MADS-IX and the Spectrum Green labeled centromere of 8 were labeled with spectrum green (Vysis, Downers Grove, IL, USA), and the chromosomes were counter-stained by 4,6-diamidino-2-phenylindole dihydrochloride.

samples from five additional patients. Because MADS-IX was mapped to chromosome 10q21, we also wanted to determine whether it was located in the region encompassing a well-known metastasis-associated gene, *PTEN* [15].

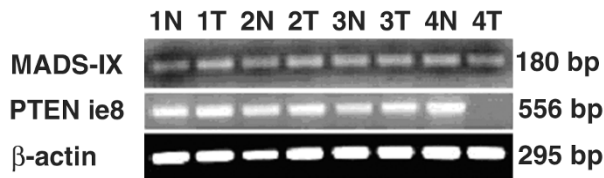
First, DNA samples from these five patients were screened by PCR using primers designed for MADS-IX

Figure 4



PCR screening of MADS-IX on normal cell DNA, primary cell DNA and metastatic cell DNA samples of five patients. PCR results show the target DNA band (180 bp) missing in patients 1 and 3. While MADS-IX showed a reduction in the intensity (about one-half) of the PCR band (heterozygous loss) in the primary tumors (P) of patients 1 and 3, it is completely missing (homozygous loss) in their metastatic (M) cell DNA samples but present in normal cell DNA (N). β -Actin was used as an internal control.

and *PTEN*. MADS-IX showed PCR amplification in normal cell DNA samples of all the five patients, but the intensities of the PCR bands were less in the primary tumor cell DNA samples and were totally undetectable in the metastatic cell DNA of patients 1 and 3 (Fig. 4). The *PTEN* gene, on the contrary, was detected, without any difference in the intensity of PCR bands in the DNA samples of normal

Figure 5

PCR screening of MADS-IX and *PTEN* on DNA samples from four tumor cell lines and matched normal DNA. Results (first row) show that MADS-IX is present in all the tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937) especially the HCC-1937 cell line, which has loss of a region encompassing the *PTEN* gene. Screening of *PTEN* in these four cell lines (second row) show its presence in the HCC-1806, HCC-1143 and HCC-1428 tumor cell lines but, as expected, it is missing in HCC-1937. β -Actin was used as an internal control.

cells, primary tumor cells and metastatic lymph node cells of all five patients.

Second, MADS-IX and *PTEN* were screened in four breast tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937) and matched normal DNA. The first three tumor cell lines had known losses in specific chromosomal regions, and the fourth had loss of homozygosity of the *PTEN* gene. PCR screening of MADS-IX and *PTEN* showed that MADS-IX is present in all four tumor cell lines, but *PTEN* is only present in cell lines HCC-1806, HCC-1143 and HCC-1428 and is missing in HCC-1937 (Fig. 5).

Discussion

Reports show a statistically significant increase in loss of heterozygosity events involving unknown genes on chromosome 16q [26], on chromosome 10q [15] and on chromosome 14q [27], in metastatic breast cancers. Measuring loss of heterozygosity, O'Connell *et al.* [27] found that the majority of lymph node-negative primary breast tumors did not amplify a region linked to D14S62 and D14S51, while lymph node-positive breast tumors retained heterozygosity for these markers. These data could imply the existence of metastasis promoting gene(s) in that region or, alternatively, the observed molecular changes may be used as a marker of metastatic propensity [28].

In the present investigation we used RDA in an attempt to identify genetic alterations related to breast cancer metastasis by comparing normal cell DNA and metastatic cell DNA of a ductal breast carcinoma patient. Ten unique candidate MADS were identified and the sequence homologies found by searching the gene bank of these MADS are presented in Table 2. The search revealed 82–99% homology to known human gene sequences for nine of these MADS but MADS-IV was found to be novel, having no homology with the sequences in the gene

banks. Further characterization of MADS-IX revealed interesting information on the genes and sequence tagged sites (STS) markers surrounding it. Within a range of 1.4 Mb of MADS-IX, we noticed two *CDC2* genes (cell division cycle 2 protein, isoform 1 and isoform 2) and two splice variants of the *ZWINT* gene (without known exact function). In the range of 821 kb, there are nine STS markers (RH26942, SHGC-58267, SHGC-79901, AFM336XD1, SHGC-103705, SHGC-3856, SHGC-84937, STSG72171 and SHGC-81245).

Physical and cytological mapping of MADS-IX confirmed that it is located at 10q21.1. Screening of MADS-IX on DNA samples from five more sets of normal, primary and matched metastatic tumor samples (Fig. 4) revealed that MADS-IX is lost in the transition from normal cells to primary cells to metastasis cells in two of five cases (three of six cases if the case used in the RDA assay is included). PCR screening of *PTEN* on these five patient samples and screening of MADS-IX and *PTEN* on a breast carcinoma cell line that had loss of homozygosity of the *PTEN* gene revealed that MADS-IX is neither a part of the *PTEN* gene or localized in the homozygous loss region of chromosome 10q encompassing the *PTEN* gene. These findings together strongly suggest that MADS-IX is a candidate marker for a novel metastasis suppressor gene. Additional archival cases are being screened to determine whether MADS-IX is a reliable marker for metastasis in ductal breast cancers.

Competing interests

None declared.

Acknowledgments

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Correspondence

Patnala Mohan R Achary, Metastasis Laboratory, Ullmann Building-1219, Department of Radiation Oncology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA. Tel: +1 718 430 2699; fax: +1 718 430 2454; e-mail: achary@aecom.yu.edu