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Original Article

ORIGINAL ARTICLE

In bone metastasis miR-34a-5p absence inversely correlates with Met expression, while Met oncogene is unaffected by miR-34a-5p in non-metastatic and metastatic breast carcinomas

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Abstract

The highlight of the molecular basis and therapeutic targets of the bone-metastatic process requires the identification of biomarkers of metastasis colonization. Here, we studied miR-34a-5p expression, and Met-receptor expression and localization in bone metastases from ductal breast carcinomas, and in ductal carcinomas without history of metastasis (20 cases). miR-34a-5p was elevated in non-metastatic breast carcinoma, intermediate in the adjacent tissue and practically absent in bone metastases, opposite to pair-matched carcinoma. Met-receptor biomarker was highly expressed and inversely correlated with miR-34a-5p using the same set of bone-metastasis tissues. The miR-34a-5p silencing might depend on aberrant-epigenetic mechanisms of plastic-bone metastases, since in 1833 cells under methyltransferase blockade miR-34a-5p augmented. In fact, 1833 cells showed very low endogenous miR-34a-5p, in respect to parental MDA-MB231 breast carcinoma cells, and the restoration of miR-34a-5p with the mimic reduced Met and invasiveness. Notably, hepatocyte growth factor (HGF)-dependent Met stabilization was observed in bone-metastatic 1833 cells, consistent with Met co-distribution with the ligand HGF at plasma membrane and at nuclear levels in bone metastases. Met-protein level was higher in non-metastatic (low grade) than in metastatic (high grade) breast carcinomas, notwithstanding miR-34a-5p-elevated expression in both the specimens. Thus, mostly in non-metastatic carcinomas the elevated miR-34a-5p unaffected Met, important for invasive/mesenchymal phenotype, while possibly targeting some stemness biomarkers related to metastatic phenotype. In personalized therapies against bone metastasis, we suggest miR-34a-5p as a suitable target of epigenetic reprogramming leading to the accumulation of miR-34a-5p and the down-regulation of Met-tyrosine kinase, a key player of the bone-metastatic process.

Introduction

Met, the product of c-*met* proto-oncogene, is the hepatocyte growth factor (HGF) receptor with a multifunctional docking site (1). Morphogenic and invasive functions are played by Met during embryogenesis, and in the development of various tissues including mammary gland (2–4). Met triggering by HGF is critical for hepatic regeneration, while the deregulation of HGF-Met signalling occurs in carcinomas including that of the liver (5–8). Different data indicate that the expression of HGF-Met couple is higher in breast carcinoma than in benign lesions (9), index of advanced stage and poor prognosis (1,10), or that Met immunoreactivity is more frequent in grade 1 compared with grade 3 cancers (11). Met confers aggressive behaviour to the *in situ* ductal carcinoma (DCIS) (4).

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Abbreviations	
DCIS	*in situ* ductal carcinoma
HGF	hepatocyte growth factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide
TMA	tissue microarray.

Met-oncogene activation in tumours depends on gene amplification, germline or somatic mutation, and receptor overexpression, and on additional arrays of molecular mechanisms such as epigenetic modifications: hypomethylation of Met promoter occurs in pancreatic ductal adenocarcinoma; methylation of long interspersed nuclear element and of the Met inhibitor IncRNA Meg3 enhances Met expression in hepatocarcinoma and insulinoma, respectively (12). In the 1833-xenograft model of bone metastasis, HGF/Met axis is regulated by DNA methylation, and its blockade leads to a delay of metastasis colonization (13), but we do not know whether their gene promoters or noncoding RNAs (miRNAs) are affected. A similar inhibitory effect on metastasis outgrowth was obtained with NK4 (competitive inhibitor of HGF) combined with Src-tyrosine kinase inhibition (14). HGF/Met couple activates Akt and Src in parental invasive MDA-MB231 cells and in 1833-bone metastatic clone (14,15). Notably, Src may influence directly Met function (16) explaining the partial independence of Src activity from HGF in 1833 cells (14). Since Akt and Src participate in the activation and stabilization of HIF-1 and Ets1 transcription factors (17,18), with consensus sequences on Met promoter (12,19), an autoregulatory loop for Met-receptor expression is triggered under HGF.

In breast carcinoma and bone metastatic cells specific functions of Met seem to depend on the nuclear localization (13,20), but data are lacking in human specimens of primary-breast carcinoma and the corresponding bone metastasis. Met transported by exosomes instructs the secondary site in the case of melanoma metastatization (21), and exosomal HGF influences the paracrine pattern between supportive cells-bone metastatic cells (13).

About 30 miRNAs regulate Met expression, and they seem to inversely correlate with protein receptor amount (12). Interestingly, some of these miRNAs including the miR-34a-5p may be epigenetically silenced (22). Notwithstanding miRNA involvement in different steps of the metastatic process is suggested (23), in humans the pattern of miR-34a-5p during breast carcinoma formation and progression to bone metastasis has never been investigated in relation to Met expression. miRNA mutations may affect the complementarity with the targets (24); a single miRNA can regulate multiple targets, involved in interconnected signalling pathways, such as Akt and Src controlled by miR-34a-5p (25,26).

Scarce is the knowledge of Met function in human bone metastasis from ductal breast carcinoma, as well as the comparison with the primary tumour as regards the molecular mechanisms involved in the regulation of Met expression. The highlight of these aspects would be critical for epigenetic therapies targeting Met and the metastasis-microenvironment cross-talk. The methylation-dependent silencing of miRNAs might have profound effects through their influence on target genes, and molecular pathways regulated by them. The network of microenvironmental signals is important for bone metastasis colonization, and HGF may influence DNA methylation (27). Metastases are characterized by high plasticity, and DNA methylation intervenes in phenotype adaptability to the different environments envisaged during dissemination, engraftment and colonization at the secondary bone site (28,29).

In this article, we examined the expression of Met receptor in human pair-matched ductal breast carcinoma and bone metastasis, and the relationship with miR-34a-5p: the aim was to evaluate whether a different control of Met occurred through miR-34a-5p expression during carcinoma progression. We hypothesize a differential involvement of DNA methylation in miR-34a-5p expression, and various roles played by HGF and miR-34a-5p on Met accumulation. Using multisample tissue microarray (TMA), we also examined miR-34a-5p in non-metastatic ductal breast carcinoma, the adjacent tissue, normal mammary gland and bone metastasis. This approach has the potential to reveal novel insight into the mechanisms of cancer metastasis to skeleton, and can be optimized for clinical use in personalized management of bone metastasis.

We observed that miR-34a-5p was highly expressed in ductal breast carcinoma without and with bone-metastasis outcome, while being practically absent in bone metastases and in normal mammary gland. Met accumulation occurred principally in primary non-metastatic carcinoma and bone metastasis, but the regulation of Met expression differed in these conditions. In primary carcinoma without history of metastasis, miR-34a-5p seemed inactive permitting transactivation of Met; an inverse correlation instead occurred between miR-34a-5p and Met in bone metastasis. Also, Met and HGF co-localized at membrane level and in nuclei of bone metastases, suggesting a critical regulation of the axis by HGF originating from microenvironment and metastatic cells. miR-34a mimic and DNA-methyltransferase inhibition reduced Met-protein level indicating their regulatory role in the expression of Met receptor in bone metastatic cells, key for migration.

Materials and methods

Observational subjects and immunohistochemistry

Bone metastases (n = 5) were collected during surgical interventions at Istituto Ortopedico Galeazzi-IRCCS, Milano, Italy, and the pair-matched invasive ductal-breast carcinomas (ER/PR positive and HER2 positive or negative) were furnished by the Hospital site of the surgery. We examined also ductal breast carcinomas (n = 5) without history of metastases (interventions at Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy). The informed consent of the patients was obtained in accordance with Declaration of Helsinki. Normal mammary gland slides were from US Biomax (Rockville, MD, USA).

The specimens were fixed, and decalcification was performed for bones (14). We immunostained three serial sections with anti-Met (C-12) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-αHGF (H487) (3 µg/ml, IBL, Aramachi, Takasaki-Shi, Gunma, Japan) or anti-E-cadherin (clone 36) (1:100, Transduction Laboratories, Bedford, MA, USA) antibody.

TMA and *in situ* miRNA hybridization

TMA preparations were histology slides (BC08032 US Biomax) containing 21 cores for each of the following specimens, i.e. normal mammary gland, invasive-ductal breast carcinoma and the adjacent tissue. Using specimens collected at Istituto Ortopedico Galeazzi-IRCCS, we constructed a TMA with cores from 18 different cases of bone metastases (each sample in triplicate), and histology slides were prepared (Azienda Ospedaliera San Gerardo, Dipartimento di Chirurgia e Medicina Traslazionale, Università degli Studi di Milano-Bicocca, Monza, Italy). To guide the samplings for the construction of the bone metastasis microarray, sections were cut from the donor block embedded in paraffin, and were stained with standard hematoxylin–eosin method. This permitted to choose morphologically representative regions of the paraffin-embedded samples of bone metastasis, which were cored at 1 mm diameter in triplicate, and were transferred to a recipient paraffin block. After the construction of the array block, serial sections (4 µm) were cut and used for the *in situ* miRNA hybridization.

Also, we used serial sections in triplicate from two patients operated first for invasive-ductal breast carcinoma, and secondarily for bone metastasis.

In situ detection of miR-34a-5p was performed with miRCURY Locked-nucleic Acids (LNA™s) ISH optimization kit from Exiqon (Exiqon, Vedbaek, Denmark), following the manufacturer's instructions. We used as probes 5′–3′ double-digoxigenin labelled complementary miR-34a-5p, 5′ digoxigenin labelled RNU6B (positive control) and scramble (negative control) oligonucleotides. Tissue slides were incubated with 80 nM miR-34a-5p and the scramble probes, or 25 nM RNU6B for 1 h at 50°C below the calculated melting temperature of the probe. After washes at hybridization temperature, bound probes were detected by enzyme-coupled antibodies (AP conjugated anti-digoxigenin Fab fragments from Roche Mannheim, Germany), followed by colour reaction with the NBT/BCIP reagent (Roche). We performed counterstaining with nuclear-fast red staining solution (Sigma-Aldrich, Saint Louis, MO, USA), and microscopy analysis under Eclipse E-1000 equipped with a Dxm 1200 digital camera (Nikon, Milano, Italy). A Nikon ACT-1 program was used for image acquisition.

Cells and treatments

The 1833 bone-metastatic clone and the parental MDA-MB231 breast carcinoma cells were kindly given by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York). Non-invasive MCF-7 cells were from European Cell Cultures Collection (Salisbury, UK). The comparative study of transcriptome profile of 1833 and MDA-MB231 cells identifies a gene set whose expression pattern is associated with, and promotes the formation of, metastasis to bone (30). 1833 and MDA-MB231 cells were authenticated with the method of short-tandem repeat profiling of nine highly polymorphic short-tandem repeat loci plus amelogenin on September 2014 (Cell Service from IRCCS-Azienda Ospedaliera Universitaria San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). The MDA-MB231 and 1833 cells were routinely maintained in DMEM containing 10% fetal bovine serum (Sigma-Aldrich), and the MCF-7 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. All the cells were used after two or three passages in culture (14).

miR-34a mimic or inhibitor transfection

The cells were transfected with 20 nM chemically modified double-strand RNA that mimics endogenous miR-34a-5p (*mirVana*® miR-34a), or with 30 nM single-strand RNA molecule designed to specifically bind to and inhibit endogenous miR-34a-5p (*mirVana*® miR-34a inhibitor) (ThermoFisher Scientific Walthman, MA, USA) (31,32). All transfections were performed using Lipofectamine 2000® (ThermoFisher Scientific), according to the manufacturer's instructions. After 48 h, the transfected cells were used as follows.

Immunoprecipitation and Western blot assays

(i) The starved cells were exposed to 100 ng/ml HGF (R&D System, Abingdon, UK); total protein extracts were prepared, and samples (1 mg) were used for immunoprecipitation with 6.5 µg of anti-Met antibody (20). (ii) Some cells were pretreated for 2 h with 100 µg/ml cycloheximide before HGF exposure, and protein extracts were prepared at various times thereafter; (iii) some cells were transfected with miR-34a mimic or inhibitor, or were exposed to 5 µM 5-aza-2′-deoxycytidine (dAza) for 1 day (13): samples (100 µg of protein) were used for Western blot analyses. Hybridization was performed with the antibody anti-Met (1:200) or anti-phosphoMet 1234/35 (1:2000, BD-Transduction Laboratories, Franklin Lakes, NJ, USA). Densitometric analysis was performed after reaction with ECL plus chemiluminescence kit from Thermo-Fisher Scientific, Pierce.

MTT assay

Cell growth determination kit based on 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) (Sigma Aldrich) required the removal of the culture medium from miR-34a mimic or inhibitor transfected cells, and the addition of MTT (10%) to fresh medium without serum for 2 h at 37°C, according to the manufacturer's instructions. The absorbance of the MTT-formazan products was measured at 570 nm using a reference wavelength at 690 nm, to subtract the background.

Cellular expression of endogenous miR-34a-5p

For miR-34a-5p detection, total miRNA was extracted from cultured cells using the mirVana miRNA isolation kit (ThermoFisher Scientific Ambion), according to the manufacturer's instructions. Complementary DNA was synthesized from 2 µg of total miRNA using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific Applied Biosystems). The expression of miR-34a-5p was assessed with qRT-PCR using Power SYBR® Green (ThermoFisher Scientific Applied Biosystems). The relative expression of miR-34a-5p was calculated using the threshold cycle (Ct), and normalized with respect to the U6 small nuclear RNA.

Matrigel invasion assay

miR-34a mimic or inhibitor transfected cells and the corresponding control cells (cultured for 48 h) were seeded (8×10^4 per well) in the upper chambers of Matrigel invasion system (BD Biocoat Cellware, Beckton Dickinson Labware, Bedford, MA, USA). The lower chambers contained the culture medium without serum. After 22-h incubation in a humidified tissue culture incubator, non-invading cells were removed from the top, and invading cells were stained with Diff-Quick (Dade Bering, Switzerland). Ten fields were randomly selected, and the number of the cells in each field was counted under 200× magnification (15).

Statistical analysis

The statistical evaluation of semi-quantitative data, Western-blot densitometric values, cell viability and migration data, and miR-34a-5p endogenous values was performed by analysis of variance. $P < 0.05$ was considered significant.

Results

HGF/Met axis in human specimens of bone metastasis pair-matched with ductal breast carcinoma, and in non-metastatic ductal breast carcinoma

In this article for the first time, we examined Met and HGF expression in human bone metastases pair-matched with primary breast carcinomas (invasive ductal, containing areas of dysplasia), and the comparison with mammary gland and non-metastatic ductal breast carcinoma was performed (Figures 1 and 2). We carried out these experiments to evaluate the importance of HGF/Met-receptor axis in the metastatic process to the skeleton in humans. The scarce investigation of bone metastases depends on the fact that these specimens are very difficult to obtain, because patients do not usually undergo surgery. However, it would be really important for therapeutic purposes to deepen the knowledge of HGF/Met signalling pathway, and the altered regulation during breast carcinoma progression.

As resulted by the semi-quantitative evaluation of the immunohistochemistry staining (Figure 1a), Met expression was 3.4-fold higher in bone metastasis than in pair-matched ductal breast carcinoma, the latter showing a score value similar to that of the adjacent tissue. Thus, positivity for Met expression (moderate signal) was observed in 60% of the adjacent-tissue cases versus 20% of the metastatic-carcinoma cases; the mammary gland was negative for Met, as reported in the literature (4). Met total staining resulted 2.6-fold higher in non-metastatic ductal breast carcinoma than in ductal breast carcinoma with history of metastasis.

Figure 1b shows HGF signal in mammary gland, which was strong in the stromal cells and less in the epithelial cells.

a

	MET staining					
	- negative %	+ weak signal %	++ moderate signal %	+++ strong signal %	++++ very strong signal %	Total staining
mammary gland	100					0
adjacent tissue	20	20	60			140
pair matched ductal breast carcinoma	20	60	20			100
bone metastasis			20	20	60	340*
ductal breast carcinoma without history of metastasis			40	60		260 *

b

	HGF staining in mammary gland					
	- negative %	+ weak signal %	++ moderate signal %	+++ strong signal %	++++ very strong signal %	Total staining
epithelium		40	60			160
stromal cells				20	80	380**

Figure 1. Semi-quantitative evaluation of immunohistochemistry assays of Met performed by using specimens of ductal breast carcinomas, of the adjacent tissue and of bone metastasis, as well as HGF score in cell components of mammary gland. The score system used (negative, weak, moderate, strong, very strong) permitted to obtain the Total staining (number of cases = 5) by adding the values of incidence percentage multiplied for the number of +. The statistical analysis was performed with analysis of variance. At this end, we analyzed all the data in (**a**) or in (**b**), considering negative = 0, and the number of the + (1, 2, 3, 4) for all the cases. The experiments were performed three times. For Met (a), *P < 0.05 versus pair-matched ductal breast carcinoma. For HGF (b), **P < 0.005 versus the epithelium.

Figure 2 reports representative images of Met and HGF immunohistochemistry assays. Met scores relative to these images, together with those of the Supplementary 1 and 2, available at *Carcinogenesis* Online, were used for the Table (Figure 1a).

As shown in Figure 2a, mammary gland epithelium was positive only for HGF but not for Met. In low grade ductal breast carcinoma (without history of metastasis) Met and HGF showed the same score values at intracellular level, including nuclei, which are reported under the panels; considering the microenvironment, HGF was present in supportive cells (sc, 3+) and in the adjacent duct epithelium (ad, 1+). All the specimens examined gave similar results for Met and HGF (Supplementary Figure 1, available at *Carcinogenesis* Online; negative controls did not show specific signal).

Now we describe bone metastasis and the pair-matched ductal breast carcinoma (Figure 2a and b). In bone metastasis, the intensity of Met staining was elevated (patients 6 and 7): Met in metastatic cells localized mostly at plasma membrane/cytosol level and also in nuclei; a certain positivity for Met was observed in cellular and non-cellular components of the matrix. Differently, in pair-matched ductal breast carcinoma (high grade) cytosolic/membranous Met presented a lower score than in bone metastasis (patients 6 and 7); see Supplementary Figure 2, available at *Carcinogenesis* Online, for Met signal in patients 8–10.

As shown in Figure 2a, in bone metastasis also HGF showed an elevated score value, while in carcinoma bulk the cytosolic HGF scored weakly as mammary gland. See Supplementary Figure 1c, available at *Carcinogenesis* Online, for the comparison of HGF signal between high-grade metastatic and low-grade non metastatic breast carcinomas: in the latter HGF was remarkably expressed. The production of HGF by breast carcinoma cells is consistent with literature (4,9).

In the adjacent tissue (Figure 2a), Met staining localized at the apical membrane of the cells lining the tubule: a gradient of progression from left to right towards an aggressive DCIS was observed. The epithelial cells lining the dysplastic tubule scored 4+ for Met and also for HGF. All the specimens examined gave similar results. Negative control did not show specific signal.

Our data in bone metastasis and pair-matched ductal carcinoma of the breast indicated differences in the intracellular localization of the couple members: HGF/Met co-expression was observed mostly in metastatic cells, consistent with the HGF-dependent regulation of membrane and nuclear Met transactivating activity (13). HGF seemed to have autocrine and paracrine patterns in skeletal metastases. Also, we suggest the role of cellular and soluble HGF in metastasis and carcinoma stroma (Supplementary Figure 3a, available at *Carcinogenesis* Online), and some cells of bone-metastasis stroma also expressed Met consistent with Peinado *et al.* (21). Bone metastases but not pair-matched ductal breast carcinomas expressed E-cadherin, index of the epithelial phenotype (Supplementary Figure 3b, available at *Carcinogenesis* Online) (29).

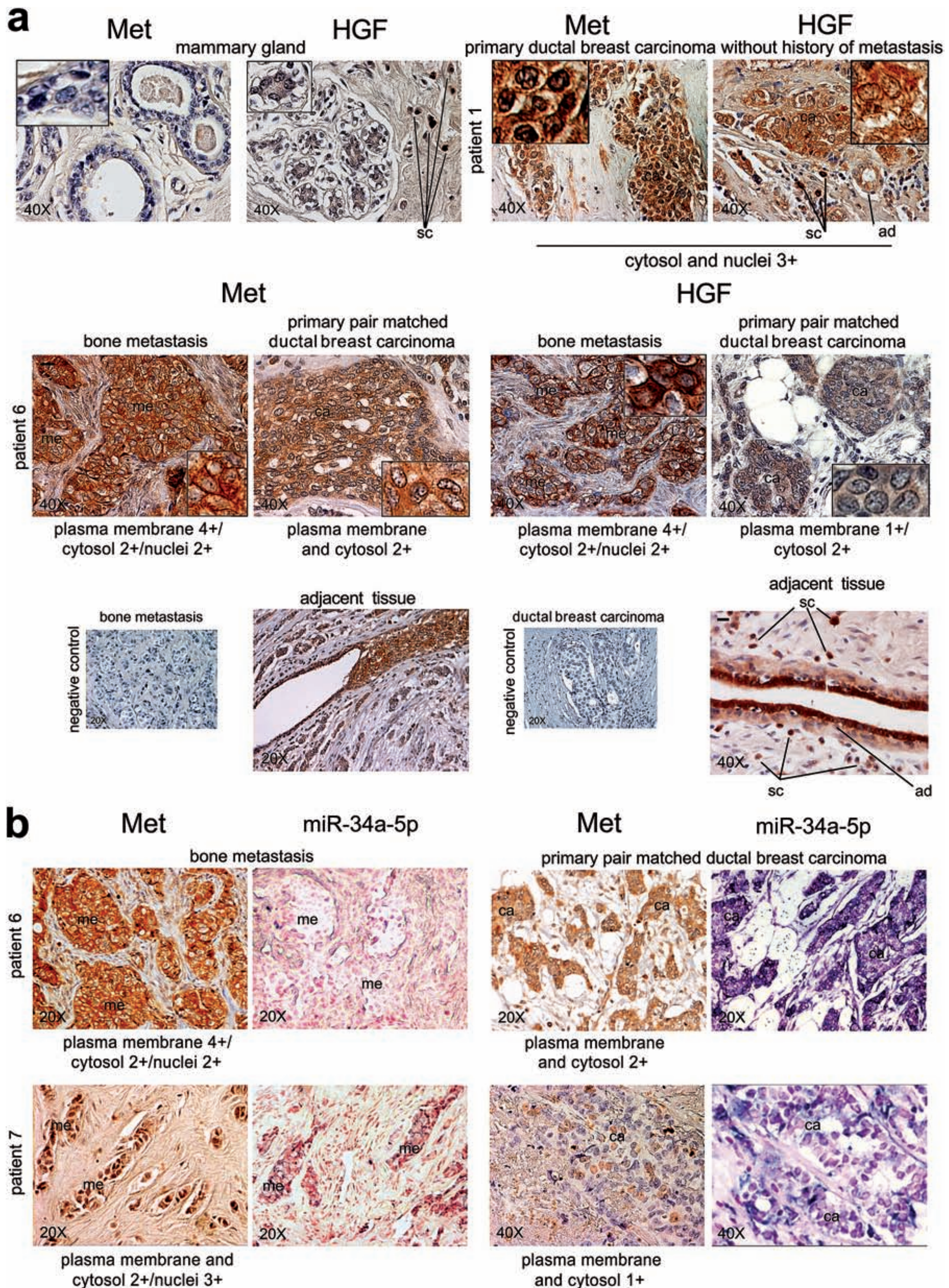


Figure 2. Met and HGF expression in non-metastatic ductal breast carcinoma, and in ductal breast carcinoma pair-matched with bone metastasis: comparison of Met with miR-34a-5p signals. The tissue sections were hybridized with anti-Met or anti-HGF antibody, or with miR-34a-5p probe. Representative images (**a**) for Met and HGF in specimens ($n = 5$), and (**b**) for Met and miR-34a-5p in serial sections of pair-matched samples (experiments repeated three times). Under the panels, the scores of Met and HGF at intracellular levels are shown. sc, stromal cells; ca, invasive ductal breast carcinoma; ad, adjacent duct; me, bone metastasis. Scale bar=120 μm (see Met immunohistochemistry in bone metastasis). Insets, magnification of details.

Moreover, in Figure 2b we show the results of reactions with Met antibody and miR-34a-5p probe in serial sections of bone metastasis and pair-matched ductal breast carcinoma. miR-34a is considered a tumour suppressor whose expression depends on methylation state (22), and it controls different targets including Met (33). The miR-34a-5p staining was remarkably positive in carcinomas versus corresponding metastases, notwithstanding moderate (patient 6) and weak (patient 7) Met signals were observed in the carcinoma specimens. The reactions with scrambled (negative control) and RNU6B (positive control) probes are shown in Supplementary Figure 4, available at *Carcinogenesis* Online.

Evaluation of miR-34a-5p expression during breast carcinoma progression

We thought interesting to evaluate whether the role of miR-34a-5p differed in bone metastasis and in ductal carcinoma without history of metastasis. We extended the study using TMA, considered a valuable tool for investigating miR-34a-5p in a large number of specimens (34). We compared miR-34a-5p expression in normal mammary gland, in invasive non-metastatic ductal breast carcinoma, in the adjacent tissue, and in bone metastases. The knowledge of miR-34a-5p pattern would permit to clarify some molecular events underlying Met expression during breast carcinoma progression.

The representative images (at 2.5× and 20×) showed that miR-34a-5p staining was elevated in non-metastatic ductal breast carcinoma opposite to bone metastasis and mammary gland (Figure 3a).

Figure 3b shows the miR-34a-5p score (negative, weak signal 1+, moderate signal 2+ and strong signal 3+). The evaluation of the score staining was performed by microscope observation of the TMA, which has been stained with the specific miR-34a-5p probe, versus TMA undergone the reaction with the scrambled probe. Using these data, we calculated the total staining, which indicated a progressive increase of miR-34a-5p expression, i.e. in the adjacent tissue and in the ductal breast carcinoma the values were 2-fold and 4-fold higher than those of normal mammary gland. In bone metastasis, the miR-34a-5p value was identical to that of the normal-breast tissue. The percentage of these score values for each kind of human specimen has been also reported in the graphic (Figure 3c).The TMA-staining reactions with specific (miR-34a-5p) and unspecific (scramble) probes, and the positive controls with RNU6B probe are shown in Supplementary Figures 5 and 6, available at *Carcinogenesis* Online.

Of note, 71% of the non-metastatic breast carcinomas and 43% of adjacent tissue were 2+ or 3+ for miR-34a-5p staining, while only 10% of mammary gland specimens showed these scores. We considered the positivity from moderate signal (2+). The latter finding is of great importance, suggesting that in the tissue surrounding the primary carcinoma miR-34a-5p undergoes early dysregulation, which might influence tumour growth affecting gene expression.

Molecular mechanisms of Met regulation by HGF, miR-34a mimic or inhibitor

In Figures 4 and 5, we studied the HGF dependent regulation of Met *in vitro* using 1833-bone metastatic clone and parental MDA-MB231 breast carcinoma cells, which are highly invasive but scarcely metastatic (13). As shown in Figure 4a, in 1833 cells between 15 and 60 min after HGF exposure, we observed a huge enhancement of pMet/Met ratio which decreased thereafter, persisting elevated until 24 h. In contrast, Met down-regulation was observed starting from 15 min after HGF exposure in MDA-MB231 cells, leading to the fall-down of pMet/Met ratio. Thus, Met in the phosphorylated form seemed stabilized by HGF in 1833 clone compared to MDA-MB231 cells.

To deepen the knowledge, we evaluated Met half-life under HGF after pretreatment with cycloheximide (Figure 4b). Under blockade of *de novo* protein synthesis, Met showed a 6-fold stabilization in HGF-treated 1833 cells versus HGF-treated MDA-MB231 cells: Met half-lives were 60 versus 10 min, respectively.

The *in vitro* data suggested that the accumulation of Met protein was influenced by HGF, explaining the histological findings in bone-metastasis specimens.

Another mechanism implicated in Met accumulation in osseous metastases might be related to miR-34a-5p expression. To modulate miRNAs there are currently two strategies: the first one is therapeutically restore the suppressed miRNA level by miRNA mimic (agonist), and the other is to inhibit miRNA function using an anti-miRNA (antagonist), that suppresses overactive miRNA function. The comparative study in non-invasive MCF-7 cells, and in bone metastatic 1833 cells showed that the transfection of miR-34a mimic or inhibitor did not affect cell survival; in parental MDA-MB231 cells the miR-34a inhibitor decreased proliferation of about 35% (Figure 4c). In fact (Figure 5a), MDA-MB231 cells showed the highest level of endogenous miR-34a-5p, which was 2-fold that of MCF-7 cells and almost 10-fold that of 1833 clone. The data of miR-34a-5p levels in MDA-MB231 and MCF-7 cells were consistent with the literature (31,32). miR-34a-5p levels were very low in 1833 cells in agreement with the specific-TMA staining observed by us in bone-metastasis specimens.

In Figure 5b and c, we report that in 1833 cells the miR-34a mimic strongly reduced cell invasiveness as well as Met protein level; the latter effect was similar to that shown for dAza, inhibitor of DNA methyltransferases. Also, dAza increased the expression of endogenous miR-34a-5p (Figure 5d), leading to suggest an indirect regulatory effect of DNA methylation on Met expression in 1833 cells. For MDA-MB231 cells, the effects on Met-protein levels and cell invasiveness after miR-34a mimic and dAza were opposite to those observed for 1833 cells. The miR-34a inhibitor was practically ineffective on cell migration and Met-protein level in both the cell lines.

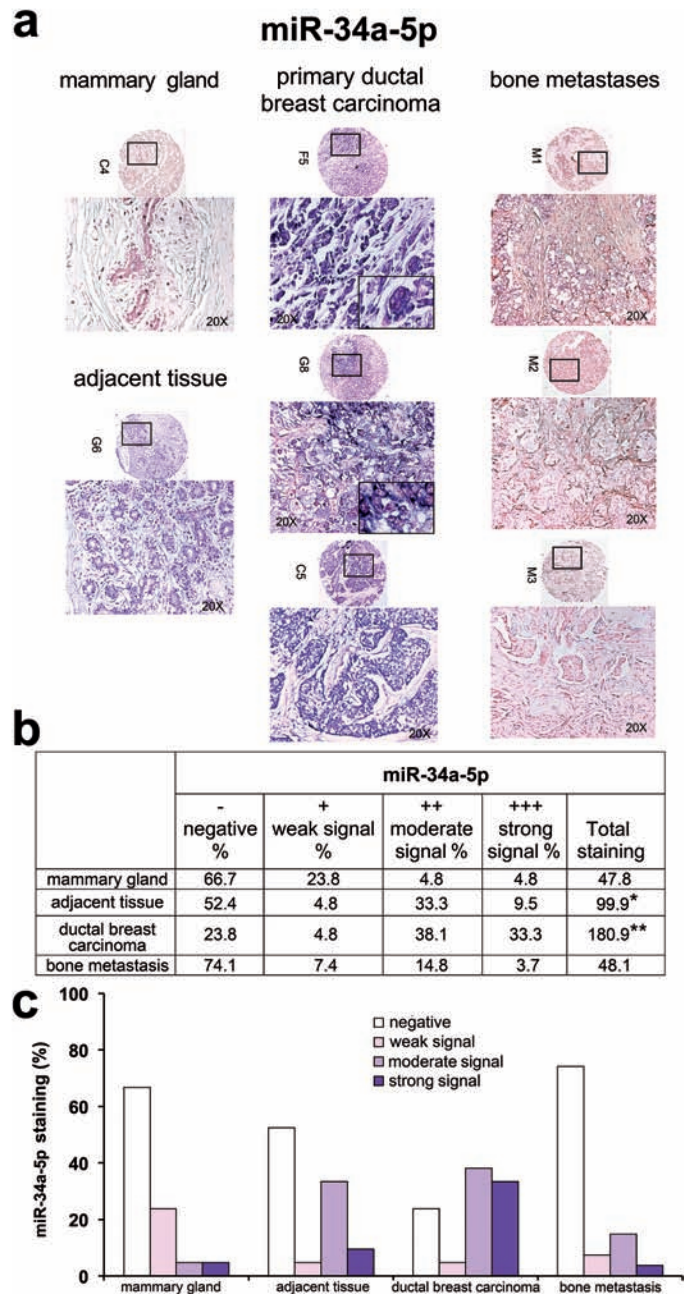
These *in vitro* data seemed to explain the differences of miR-34a-5p function towards Met in bone metastases and ductal breast carcinomas in humans: the inverse correlation miR-34a-5p/Met occurred only in bone metastases, while in low and high grade carcinomas no association miR-34a-5p/Met seemed to occur.

Discussion

The present article deals for the first time with the relationship between Met receptor and miR-34a-5p expression in breast carcinoma progression to bone metastasis, since miR-34a-5p is considered one of the miRNAs which regulate Met expression (33). However, miR-34a-5p role in bone metastasis, and the significance as early predictive biomarker and target of therapy related to Met function are largely unknown.

Our principal finding was that endogenous miR-34a-5p strongly accumulated in human specimens of non-metastatic and metastatic ductal-breast carcinomas and supportive cells: the comparison was performed with pair-matched metastases grown in the skeleton, which resulted devoid of miR-34a-5p as normal mammary gland.

Notably, half of the samples of tissue adjacent to breast carcinoma showed positivity for miR-34a-5p signal, without evident



	miR-34a-5p				
	- negative %	+ weak signal %	++ moderate signal %	+++ strong signal %	Total staining
mammary gland	66.7	23.8	4.8	4.8	47.8
adjacent tissue	52.4	4.8	33.3	9.5	99.9*
ductal breast carcinoma	23.8	4.8	38.1	33.3	180.9**
bone metastasis	74.1	7.4	14.8	3.7	48.1

Figure 3. Images of miR-34a-5p staining for TMA of mammary gland, ductal non-metastatic breast carcinoma and the adjacent tissue (n = 21), and of bone metastasis (n = 18). (**a**) Representative images of specific miR-34a-5p staining. The patients were identified by the code of the original slides. The images were taken at 2.5× and 20× magnifications. (**b**, **c**) Semi-quantitative evaluation of miR-34a-5p staining and the statistical analysis of the data were made as in Figure 1. At this end, we analyzed all the data of the table, considering negative = 0, and the number of the + (1, 2, 3) for the cases (total number = 20). The experiments were performed three times. *P < 0.05, **P < 0.005 versus mammary gland. The score values for miR-34a-5p staining, and the percentage of incidence for the different specimens examined has been shown (graphic).

histological changes of ductal epithelium. This finding suggests that the microenvironment was influenced by carcinoma outgrowth, due to the cross-talk tumour-stroma, even if the contrary cannot be excluded. Supportive cells are likely to deliver exosomes containing miRNAs (35,36); the soluble content of microenvironment (miRNAs and growth factors) may be relevant for neoplastic disease progression and prognosis, affecting carcinoma cell phenotype through epigenetic mechanisms (37,38).

The general opinion is that miR-34a is an oncosuppressor frequently decreased in tumours, even if these studies are performed with cell lines (39), or adding exogenous miR-34a as in prostate carcinoma cells (40). Here, we showed that endogenous levels of miR-34a-5p were much more elevated in MDA-MB231 invasive breast carcinoma cells than in 1833 bone-metastatic clone, while MCF-7 non-invasive breast carcinoma cells had intermediate values.

Based on our data, miR-34a-5p was not silenced in ductal-breast carcinomas with favourable outcome (low grade) or even poor prognosis (high grade), but it seemed inactive towards Met target, so that the tyrosine kinase receptor accumulated probably via a transcriptional mechanism. Consistently, in MDA-MB231

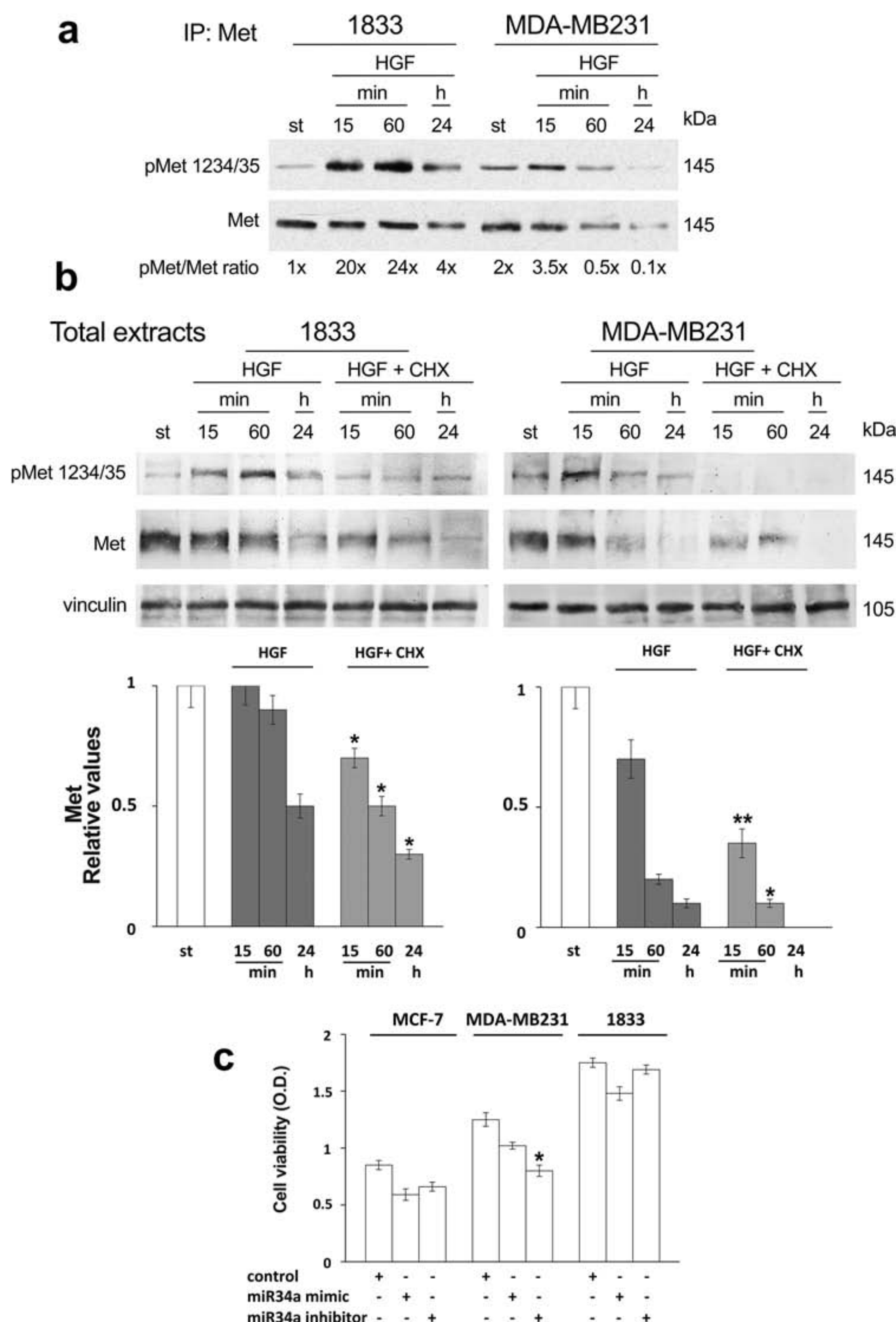


Figure 4. HGF affects Met expression, its phosphorylation and the half-life in invasive and metastatic breast carcinoma cells, and effect of miR-34a mimic or inhibitor on cell viability. (**a**) Total protein extracts were immunoprecipitated with anti-Met antibody, and analyzed by Western blot. The experiments were performed three times with similar results. (**b**) Representative images of Western blots performed with protein extracts from cells treated or not with cycloheximide (CHX) in the presence or the absence of HGF; vinculin was used for normalization. The data shown in the histograms are the means ± S.E. of three independent experiments. *P < 0.05 and **P < 0.005 versus corresponding HGF-treated cells. (**c**) MTT assay was performed after cell transfection with miR-34a mimic or inhibitor. Three independent experiments in triplicate were performed, and the data shown are the means ± SE. *P < 0.05 versus control MDA-MB231 cells.

breast carcinoma cells the restoration of miR-34a-5p with the mimic increased the Met-receptor expression and cell invasiveness, opposite to the antagonist (miR-34a inhibitor) ineffective towards the same parameters, indicating that miR-34a-5p was non overactive towards Met in this context: the proliferation of carcinoma cells was, instead, reduced by miR-34a-5p inhibitor. We hypothesize that miR-34a-5p might influence malignancy blocking stemness biomarkers, which include CD44 and Notch (33): cancer staminality is responsible for aggressive growth with formation of metastases, and resistance to therapy (41).

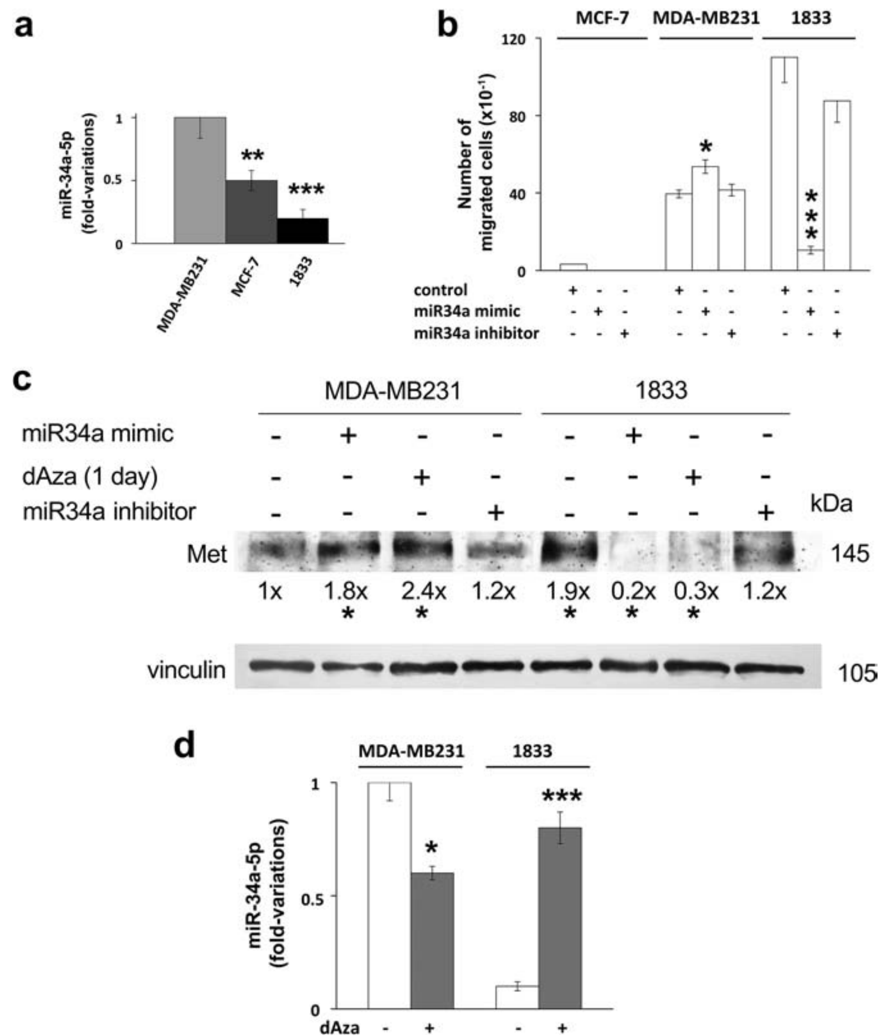


Figure 5. Endogenous levels of miR-34a-5p, and effects of miR-34a mimic or inhibitor on cell migration and Met expression: role of DNA methyltransferase blockade with dAza. (**a**) The relative expression of miR-34a-5p was measured in different cell lines: three independent experiments were performed, and the data shown are the means ± S.E. **$P < 0.005$ and ***$P < 0.001$ versus miR-34a-5p value of MDA-MB231 cells. (**b**) Matrigel invasion assay was performed after cell transfection with miR-34a mimic or inhibitor for 48 h. Three independent experiments in triplicate were performed, and the data shown are the means ± SE of 10 fields. *$P < 0.05$ and ***$P < 0.001$ versus the respective control value. (**c**) Proteins were extracted from the pellets of cells transfected with miR-34a mimic or inhibitor, or treated with dAza, and were analyzed by Western blot assay. The experiments were performed three times; vinculin was used for normalization. *$P < 0.05$ versus the first line. (**d**) miR-34a-5p was measured in cells treated or not with dAza. Three independent experiments were performed, and the data shown are the means ± SE. *$P < 0.05$ and ***$P < 0.001$ versus the respective untreated cells.

Even if the data of literature are contradictory (11), our present findings supported the high expression of Met in low grade carcinomas: in the examined specimens of non-metastatic ductal breast carcinoma Met protein was localized in the nuclei, index of specific functions related to mesenchymal phenotype.

About 65–75% of patients with breast cancer suffer from skeletal metastases, increasing the morbidity and mortality rates, due to perturbation of bone remodelling consequent to metastatic cells–bone microenvironment interaction; miRNAs are involved in homeostasis and metastatic disease of the bone (42). Specific miRNAs regulating heparanase expression affect exosome production and release of HGF in the microenvironment (43). We observed autocrine and paracrine loops for HGF in human bone metastases, and a strong co-expression with Met at membrane and nuclear levels, while miR-34a-5p was down-regulated.

Numerous explanations can be given for the function of miR-34a-5p against various targets during tumour progression; different molecular patterns and events activated in primary carcinomas and bone metastases were likely to oppositely affect miR-34a-5p expression, while Met being expressed in these specimens even if with diverse score and localization (Figure 6). (i) miRNAs play regulatory mechanisms by binding to 3′-untranslated region, responsible for transcription block, by facilitating transcript degradation and/or by suppressing the expression/function of numerous critical proteins: miRNAs may target components of cell-signalling network, as shown for Akt and Src inhibited by miR-34a-5p (25,26). In breast carcinomas, mutation and editing events might change the complementarity of miR-34a-5p for these kinases; as a consequence HIF-1 and Ets1 might transactivate Met under the HGF-dependent activation of Akt and Src (15). In fact, HGF (autocrine) was elevated especially in non-metastatic carcinomas, compared with metastatic carcinomas, concomitant with the highest Met levels. Met function and regulation has to be considered in the context of *in vivo* conditions, such as production and concentration of

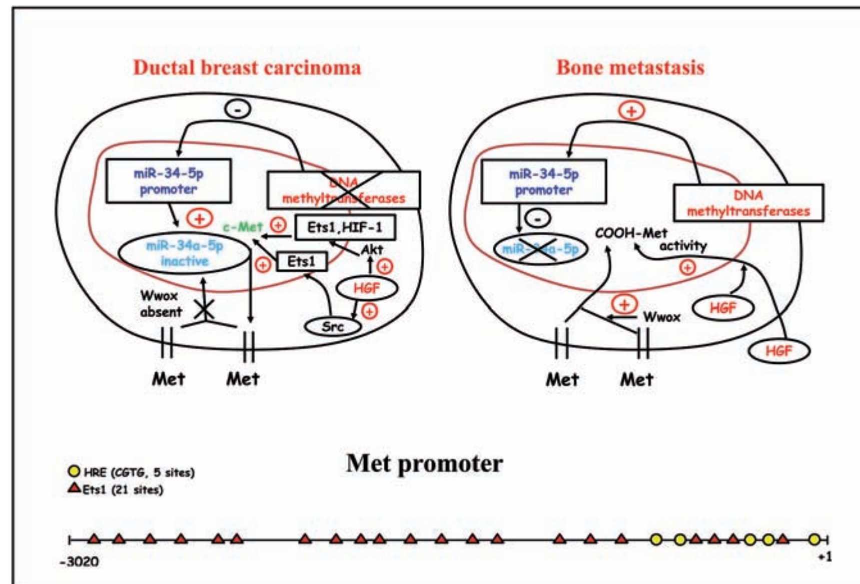


Figure 6. Regulation of the expression of miR-34a-5p and Met in ductal breast carcinoma and in bone metastasis. In bone metastasis, miR-34a-5p silencing might depend on hypermethylation of its promoter, with consequent expression of Met. Due to the presence of the transcriptional regulator Wwox in bone metastases, Met translocation in the nucleus as COOH-fragment is favoured (13). In contrast, in ductal breast carcinoma miR-34a-5p was present, but it seemed inactive towards Met: it did not interfere with the transcriptional control of Met, which might occur via HIF-1 and Ets1 regulated by Src and Akt under HGF. The consensus sites for HIF-1 (HIF-1 responsive element, HRE) and for Ets1 on the entire Met promoter are shown. In pair-matched breast carcinoma, Met receptor was expressed principally at cytosol/plasma membrane level.

HGF (originating from stroma and also from breast carcinoma cells), intercellular junctions, mesenchymal/epithelial phenotype, and methylation state. (ii) In human-bone metastases, aberration of DNA methylation underlies the plastic phenotype (28,29). Consistently, in 1833-bone metastatic clone under DNA-methyltransferase blockade miR-34a-5p level augmented with consequent decrease of Met-protein level. At support, by restoring miR-34a-5p with the mimic Met protein decreased, hampering the migration of 1833 cells. HGF stabilized the Met receptor in 1833 clone versus MDA-MB231 cells, amplifying the effect on signalling pathways downstream. Notably, HGF might influence the methylation state of bone metastases in humans, as demonstrated in the xenograft model (13), possibly affecting the accumulation of miRNAs. (iii) Complex complementary interaction between gene promoter-methylation at 5′ (transcriptional regulation) and post-transcriptional regulation at the 3′ untranslated region level seems to occur (44). This complementation is reported for different genes, with prevalence of one or the other of the two molecular mechanisms. We hypothesize that for the same gene like Met one of the two mechanisms takes the prevalence with the phase of tumour progression. Based on the *in vitro* data, in breast carcinomas the Met promoter seemed under the partial methyltransferase inhibitory function, becoming insensitive to 3′ untranslated region regulation by miR-34a-5p: this was the reason why dAza augmented Met expression. Differently, in bone metastases Met accumulation was indirectly controlled by methyltransferases, depending on miR-34a-5p down-regulation. When miR-34a-5p accumulated under dAza, Met expression was reduced through 3′ untranslated region interaction, an inverse relationship similar to that observed in the dAza-treated 1833 xenograft model (13).

The expression of miRNAs depends on a complex array of mechanisms related to intrinsic molecular characteristics of the cells, but possibly also affected by microenvironmental signals like HGF, and a stringent correlation between miR-34a promoter methylation and miR-34a-5p levels is lacking in MDA-MB231 and MCF-7 cells (45). In 1833 cells, the miR-34a-5p down-regulation might be due to methylation of the region upstream of the transcription start, preventing p53 binding, or of the precursor promoter (45). Because of the possible gain of function (46), p53 mutant in MDA-MB231 cells might transactivate miR-34a-5p, while its promoter methylation was moderate (45). Consistently, the methylation of cytosine at position 5 in CpG, which is accompanied by the inactivation of surrounding chromatin due to recruitment of histone deacetylase, is critical for the gene expression pattern (45). MCF-7 have wild-type p53 at a difference with MDA-MB231 cells (47), and miR-34a-5p may represent a mechanism by which tumour cells inactivate or weaken the check points that involve p53 (45).

Altogether, specific miR-34a-5p targets might be ancillary to the HGF/Met dependent phenotypes. Experimental evidence indicates that HGF/Met axis is important for epithelial–mesenchymal transition in primary breast carcinoma, influencing invasiveness through production of proteases and Endothelin-1 (38,48,49). Differently, in bone metastases HGF/Met axis is responsible for the adhesion/epithelial phenotype related to E-cadherin expression, and for osteolysis dependent on TGFβ transactivating activity downstream (38).

In agreement with our present findings, the most aggressive DCIS shows an imbalance of Met expression in respect to surrounding tissue (4); the high-grade dysplasia corresponds to DCIS, a biologically and clinically heterogeneous disease (50). Even if not all types of DCIS progress to invasive carcinoma, this histological pattern may represent a late stage of cell deregulation. The natural history is influenced by both tumour- and host-related factors. In DCIS patients, the radiation therapy performed after surgery approximately halves local recurrence, both *in situ* and invasive. However, it might be an ‘overtreatment’ for the subgroup of patients with low risk of progression to invasive breast cancer, for whom local surgery would be sufficient.

The concomitant expression of miR-34a-5p and Met in DCIS might represent an early predictive biomarker of worst outcome.

In conclusion, the role of the microenvironment on metastatic phenotype due to miRNA release, HGF production and DNA methylation controlled also by exosomes has paramount importance for the therapy (37,43). In numerous studies of our laboratory we suggest that therapies devised to affect the phenotype through epigenetic reprogramming are more effective and suitable to prevent metastatic growth in respect to those aimed at modifying the genotype (13). To make definitive conclusions, it would be necessary to extend the present study which considers, however, a homogeneous group of specimens of non-metastatic ductal breast carcinoma and of bone metastases.

The patient stratification according to HGF/Met receptor expression needs further development to become an important component of the study design in clinical trials, but miR-34a-5p seems to represent an early diagnostic biomarker, that when associated with elevated expression of HGF/Met couple would indicate poor prognosis. The blockade of DNA methylation prolongs the survival of the xenograft mice (13), and might be an innovative approach to fight bone metastases by increasing miR-34a-5p and down-regulating Met in humans.

Supplementary material

Supplementary date are available at *Carcinogenesis* online.

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