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Molecular alterations of E-cadherin and beta-catenin in brain metastases

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1. ABSTRACT

The molecular mechanisms and candidate genes involved in metastasis to the brain need elucidation. In the present study brain metastases were analyzed regarding changes of E-cadherin (CDH1) and beta-catenin (CTNNB1). Loss of heterozygosity (LOH) of the CDH1 gene was detected in 42.2% of samples. The highest frequency of LOHs was observed in metastases from primary sites of lung adenocarcinoma and small cell lung cancer. Metastases from breast and colon demonstrated changes in 55.6% and 50% of cases. Downregulation of E-cadherin protein was observed in 83% of samples. Only 21.1% of samples with E-cadherin LOH had beta-catenin located in the nucleus. Image analysis showed that the quantities of E-cadherin and beta-catenin were significantly positively correlated (P = 0.008). Changes of E-cadherin were frequent in brain metastases that we investigated. Lack of mutations of beta-catenin, the fact that it was not frequently found in the nucleus and the positive correlation between the two proteins may suggest that the break-up of adherens junctions, and not the activation of wnt signaling, is responsible for metastasis formation.

2. INTRODUCTION

Metastasis happens when cancer cells, having accumulated a substantial number of genomic changes, adapt to the microenvironment of an organ distant from the primary tumor and develop secondary tumors. This special feature of tumor cells of different origins, their ability for metastatic colonization, is a highly selective process conditioned by genetic profile of the original tumor as well as by the organ environment. The susceptibility of brain to secondary formation is a well known phenomenon. On the contrary, the molecular basis for invasion and metastasis to the brain is largely unknown. The incidence rates for brain metastases demonstrate that metastatic tumors are the most frequent neoplasm of the central nervous system (1, 2). Moreover, the development of intracerebral metastases is associated with high morbidity.

The work presented in this paper focused on changes of E-cadherin (CDH1) and beta-catenin genes and proteins in a set of 47 brain metastases. Loss of function of E-cadherin tumor-suppressor protein correlates with increased invasiveness and metastasis of tumors, resulting

in it being referred to as the "suppressor of invasion gene" (3). E-cadherin is one of the most important molecules of cell-cell adhesion in different tissues. It interacts with the actin cytoskeleton through linker molecules, alpha- beta- and gamma-catenins. Besides its role in cellular architecture, E-cadherin has a role in wnt signaling too (3-5). Classical wnt signaling is, through several cytoplasmic relay components, transduced to beta-catenin, which then enters the nucleus to activate transcription of target genes among which c-myc and cyclin D1. The main signaling molecule of the pathway is beta-catenin, while proteins APC (adenomatous polyposis coli) and axin act as critical components of the beta-catenin destruction machinery. When wnt ligand is absent, beta-catenin is being destroyed. In response to wnt signaling, or under the circumstances of mutated APC, beta-catenin is stabilized, accumulates in the cytoplasm and enters the nucleus. E-cadherin is an indirect modulator of wnt signaling. Since it binds to and sequesters cytoplasmic beta-catenin it modulates the cytoplasmic accumulation of beta-catenin and is in such a fashion involved in the modulation of the signal.

It has been well documented that wnt genes, together with other components of wnt signaling pathway, are implicated in tumorigenesis (6) and lately also in brain tumorigenesis (7-10). Our interest in investigating E-cadherin in brain metastases stemmed from several points. Classical cadherins such as E-type and N-type are involved in forming both adherens and synaptic junctions in the nervous system. The dysfunction of E-cadherin is a common prerequisite for lung, breast, melanoma, gastric, renal and colorectal cancer cells dissemination (11-13). There are many reports (14-16) indicating that metastasis to the brain is governed by a specific set of genetic changes of which some originate from the primary tumor (17). Reports also indicate that the expression of genes may differ in the primary site in comparison to their expression in the metastasis (18, 19). Moreover, metastasis steps and time spans differ between the tumor types. The velocity of metastasis establishment may, therefore, differ according to the genes involved. The interaction between the metastatic cell and the organ in which secondary tumor will grow is a very complex area of molecular interactions (20, 21). This tissue tropism once again brings into attention the relevance of ancient hypothesis of "seed and soil". A study by Gupta *et al.* (22) demonstrated that isolated invasive cells displayed different tissue tropism and affinity for specific secondary target and this difference was based on differential gene expression patterns.

The many analyses were usually performed on primary tumors, whereas information on genetic status of distant metastases is very scarce and inconsistent. Our idea was that the analysis of distant metastases could elucidate the consequence of tumor progression in regard of its mutational complexity.

3. MATERIALS AND METHODS

3.1. Tumor specimen

Samples of 47 brain metastasis, together with autologous blood samples were collected from the Department of Neurosurgery and Department of Pathology, University Hospital "Sisters of Charity", Zagreb, Croatia. The metastasis tissues were frozen in liquid nitrogen and transported to the laboratory, where they were immediately transferred at -75°C. The peripheral blood samples were collected in EDTA and processed immediately. Using the magnetic resonance imaging (MRI) metastasis lesions were found in different cerebral regions, predominantly in the parietal and frontal regions and cerebellum. During the operative procedure the metastasis was maximally reduced using a microneurosurgical technique. The patients had no family history of brain tumors. All metastases were studied by pathologists and classified according to the WHO criteria (2). Twenty-seven patients were male and 20 female. The age of patients varied from 39 to 81 (mean age = 60.4 years). The mean age at diagnosis for males was 61.3, and for females 59.3 years. The local Ethical Committee approved our study and patients gave their informed consent.

3.2. DNA extraction

Approximately 0.5 g of metastasis tissue was homogenized with 1 ml extraction buffer (10 mM Tris HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) and incubated with proteinase K (100 μg/ml; Sigma, USA; overnight at 37°C). Phenol chloroform extraction and ethanol precipitation followed. Blood was used to extract leukocyte DNA. Five ml of blood was lysed with 7 ml distilled water and centrifuged (15 min/5000 g). The pellet was then processed as for DNA extraction from the tissue samples.

3.3. Polymerase chain reaction

PCR amplification of polymorphic regions linked to the CDH1 gene was used to test loss of heterozygosity (LOH) in brain metastases. Allelic loss at the CDH1 locus was assessed using 3 highly polymorphic microsatellite markers - D16S265, D16S398 and D16S752, which map to 16q21-22.1. D16S265 and D16S398 are CA dinucleotide repeat polymorphisms and the D16S752 polymorphic region is a tetranucleotide GATA polymorphism (GATA51G03). All markers were amplified in a total volume of 25 μl, each primer 5 pmol, 200 ng DNA, 2.5 μl 10X buffer II, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP, 0.5 U Taq (Promega, USA). PCR conditions for D16S265, D16S398: initial denaturation 1 min/95°C; denaturation, 30 sec/95°C; annealing, 1 min/55°C; extension, 30 sec/72°C; final extension, 7 min/72°C; 30 cycles. PCR conditions for D16S752: initial denaturation 3 min/96°C; denaturation, 30 sec/96°C; annealing, 35 sec/55°C; extension, 30+1 sec/72°C; final extension, 10 min /72°C; 30 cycles. Primers are listed in Table 1. PCR products were analyzed on 2% agarose gels, length of the D16S752 repeat was 102-126 bp, length of the D16S265 repeat was 89-117 bp, and length of the D16S398 repeat was 180-196 bp.

3.4. Loss of heterozygosity, replication error

Absence or significant decrease in the intensity of one polymorphic allele in metastasis compared to the heterozygous autologous blood sample was considered as LOH of CDH1 gene. Replication error or microsatellite

Table 1. Primers

Primer	Forward (5'-3')	Reverse (5'-3')
D16S752	AATTGACGGTATATCTATCTGTCTG	GATTGGAGGAGGGTGATTCT
D16S265	CCAGACATGGCAGTCTCTA	AGTCCTCTGTGCACTTTGT
D16S398	CTTGCTCTTTCTAAACTCCA	GAAACCAAGTGGGTTAGGGTC

instability (MIN or MSI) is a type of genomic instability indicating impaired cellular mismatch repair. RER (replication error)-positive (RER+) samples have bands on different positions in comparison to bands of autologous blood tissue due to a defect in the replication/repair machinery in tumor cells. The samples were electrophoresed on Spreadex gels EL 300 (Elchrom scientific, Switzerland) at 120V. Temperature of the running buffer was kept constant at 55°C. The samples were stained with SyberGold (Molecular Probes, Netherlands). All the PCR experiments were repeated twice and the LOHs were confirmed.

3.5. Heteroduplex analysis

Exon 3 of the CTNNB1 gene was screened for mutations. Heteroduplexes were formed by heating 3 µl of PCR products (tumor mixed with normal DNA) at 95 ^{o}C for 3 min, followed by incubation on ice for 20 min. About 3 µl of each sample was mixed with 7 µl of mixture of formamide and 10 mM NaOH (1:100) prior to loading to a gel. The electrophoresis was performed on the GMA gels (Elchrom Scientific, Switzerland). The temperature of the running buffer was kept constant at 9^{o} C.

3.6. Immunohistochemistry

The samples were formalin-fixed, paraffin embedded, and 4-µm thick sections were placed on Capillary gap microscope slides (DakoCytomation, Denmark). The sections were immunostained using the biotin–avidin–horseradish peroxidase method as described previously (7). The antibodies used for E-cadherin protein detection were: monoclonal mouse anti-human E-cadherin NCH-38, and for beta-catenin: mouse anti-human beta-catenin monoclonal antibody, both Dako Corporation, Carpinteria, USA**.** The secondary antibodies were biotinylated anti-mouse immunoglobulins. All chemicals were from DakoCytomation. Negative controls were samples that underwent same staining procedure with the exclusion of the primary antibodies. Cortex of the frontal part of the normal brain, as well as normal skin, kidney and colon, served as positive controls. The analysis of the labeling was performed by two independent observers, pathologists experts in the field blinded to the conditions of the experiment, on an Olympus BH-2 microscope. Weak expression was labeled as +, moderate expression as ++, and strong expression as +++.

3.7. Image analysis

Protein expression was then quantified with the aid of Image Analyzer. For each sample, the intensity of staining in a well-defined area was evaluated using image-analyzing software manufactured by Vamstec (Zagreb, Croatia). The area covered by microscopic assessment was 200 000 square micrometers (200 000 µm^2), i.e. approximately area 0,447 x 0,447 mm, with magnification X 100. The chosen area was located in the central part of the excised tissue containing most characteristics of malignant tissue. All density measurements were "calibrated" against its slide characteristics, i.e. transparency. Density was depicted as the intensity of light retained by tissue or tissue transparency and expressed in grey scale pixels ranging from 0 to 255, zero representing no transmission of light and 255 total transparency. Density in the area of metastasis location was compared with density of the normal tissue sections.

3.8. Statistical Analysis

All individuals were analyzed for the following features: PHD status, sex, age, CDH1 LOH, E-cadherin protein expression and beta-catenin protein expression and localization. Differences in the frequencies of the analyzed features were tested with the Pearson χ^2 test employing Yates correction when appropriate. Previous to that Kolmogorov-Smirnov tests were performed to test whether the collected data had a normal distribution - low significant values (less than 0.05) indicate that the distribution of the data differs significantly from a normal distribution. In instances where there are less than 50 cases the Shapiro-Wilk test is also used for the same reason. In our case the calculated significance was P=0.221 indicating that the distribution was sufficiently normal to use the Pearson's test. The collected data were also analyzed with the T-student test and Pearson's correlation when appropriate. All statistical evaluations were performed with the SPSS statistical package 10.0 (SPSS Inc., Chicago, IL, USA).

4. RESULTS

The results of our analysis showed altogether 19 metastases with LOH of the CDH1 gene out of 45 heterozygous patients (42.2%) examined by all three microsatellite markers (Figure 1). Microsatellite markers for E-cadherin gene, D16S752, D16S265 and D16S398, were highly informative with 83%, 85% and 86.5% of heterozygosity, respectively. D16S752 is a polymorphic repeat that could show 7 different allelic variants in Croatian population. When distributing LOHs according to the specific microsatellite region, D16S752 revealed 23.1% of LOHs; D16S265 revealed 26.5%; while D16S398 revealed 21.9%. When summarizing all gross deletions striking selected microsatellite regions of the E-cadherin gene, there were 25 LOHs out of 45 heterozygous patients, 55.6%. Another type of genomic instability, replication error-positive samples (RER+) was also detected in the metastases we investigated. The instability is the result of impaired cellular mismatch repair. Four out of 45 heterozygous microsatellite regions harbored RER+ phenotype (8.9%). Exon 3, the mutational hot-spot of beta-catenin, was not targeted in any of the metastases we investigated. Genomic changes and the polymorphic status of investigated genes are summarized in Table 2.

Table 2. The polymorphic status for microsatellite markers used, genetic changes of the CDH1 gene, expression of E-cadherin and beta-catenin proteins, localization of the metastasis and pathohistological diagnosis of the primary site

PATIENT NO.	LOCATION	D16S752	D16S265	D16S398	E-CADHERIN PROTEIN	BETA-CATENIN PROTEIN	PRIMARY TUMOR
1.	Cerebellum	HOMO	**LOH**	HETERO	-	N+C++	Large cell carcinoma of the lung
2.	Cerebellum	HETERO	HOMO	HETERO	+++	C++	Large cell carcinoma of the lung
3.	Frontal region	HETERO	HETERO	HETERO	++	-	Large cell carcinoma of the lung
4.	Parietal region	HETERO	ND	ND	+++	C+	Large cell carcinoma of the lung
5.	Occipital region	HETERO	HETERO	HETERO	+	C++	Large cell carcinoma of the lung
6.	Frontal region	HETERO	HETERO	HETERO	+	N++	Large cell carcinoma of the lung
7.	Occipital region	HETERO	HETERO	HETERO	++	C+N+++	Large cell carcinoma of the lung
8.	Frontal region **PR**	HETERO	ND	HOMO	+ ++	C++ C+	Large cell carcinoma of the lung
9.	Parietal region	HETERO	HOMO	HETERO	+	C+	SCLC
10.	Frontal region	HETERO	HETERO	**LOH**	-	C+	SCLC
11.	Parietal region	**LOH**	**LOH**	**LOH**	-	C++N+++	SCLC
12.	Parietooccipital region	HETERO	**LOH**	HOMO	++	C+	SCLC
13.	Cerebellum	HETERO	RER +	HETERO	-	C+	Adenocarcinoma of the lung
14.	Temporal region	HETERO	HETERO	ND	+	C+	Adenocarcinoma of the lung
15.	Parietal region	**LOH**	HOMO	ND	++	C+	Adenocarcinoma of the lung
16.	Frontal region	**LOH**	**LOH**	**LOH**	+++	C++N+	Adenocarcinoma of the lung
17.	Temporal region	HOMO	HETERO	**LOH**	++	C+	Adenocarcinoma of the lung
18.	Cerebellum	**LOH**	HETERO	HOMO	+	C+	Adenocarcinoma of the lung
19.	Parietal region	HETERO	**LOH**	HETERO	-	C++	Squamous cell carcinoma of the lung
20.	Parietal region	HETERO	HETERO	HOMO	+++	C +N++	Squamous cell carcinoma of the lung
21.	Parietal region	HETERO	HOMO	HETERO	+++	C+++	Squamous cell carcinoma of the lung
22.	Multiple metastases	HETERO	HETERO	HETERO	++	C++N+	Squamous cell carcinoma of the lung
23.	Temporal region	HETERO	HETERO	ND	+	C+++N+	Carcinosarcoma of the lung
24.	Parietal region **PR**	HETERO	HETERO	HOMO	+ ++	C+ -	Carcinosarcoma of the lung
25.	Frontotemporal region	**LOH**	HETERO	HETERO	+	C++	Invasive ductal breast cancer
26.	Medulla spinalis	HETERO	HETERO	ND	+	C+	Invasive ductal breast cancer
27.	Dura	HOMO	HETERO	ND	++	C++	Invasive ductal breast cancer
28.	Frontotemporal region	HETERO	**LOH**	HETERO	+	C++	Invasive ductal breast cancer
29.	Cerebellum	HETERO	HETERO	**LOH**	-	C+	Invasive ductal breast cancer
30.	Cerebellum **PR**	HETERO	HETERO	ND	++ ++	C++ C++	Invasive ductal breast cancer
31.	Parietal region	HETERO	HETERO	HETERO	+++	C+	Invasive ductal breast cancer
32.	Frontal region	**LOH**	ND	HETERO	-	C+++	Invasive ductal breast cancer
33.	Cerebellum	**LOH**	ND	HETERO	++	C+N+++	Invasive ductal breast cancer
34.	Cerebellum	HETERO	HETERO	HETERO	+	C+	Colon adenocarcinoma
35.	Frontal region	HETERO	HETERO	**LOH**	--	C++	Colon adenocarcinoma
36.	Parietal region	**LOH**	**LOH**	HETERO	-	C+	Colon adenocarcinoma
37.	Cerebellum	HOMO	HETERO	HETERO	+	C+	Colon adenocarcinoma
38.	Cerebellum	HOMO	ND	ND	-	-	Colon adenocarcinoma
39.	Parietal region **PR**	HETERO	HOMO	HETERO	++ ++	C+ C+	Gastric adenocarcinoma intestinal
40.	Medulla spinalis	HETERO	HETERO	HETERO	+	N+++	Gastric adenocarcinoma mixed
41.	Parietoocipital region	HETERO	HETERO	**LOH**	++	C+	Clear cell renal carcinoma
42.	Parietal region	HETERO	HOMO	ND	++	C+++ N+	Clear cell renal carcinoma
43.	Frontal region	**LOH**	**LOH**	RER+	+++	C+	Sebaceous carcinoma
44.	Cerebellum	HOMO	HETERO	HETERO	+	C+N++	ND
45.	Cerebellum	RER+	ND	RER+	++	C+N+++	ND
46.	Cerebellum	HOMO	**LOH**	HETERO	C+++	C+	Neuroendocrine carcinoma
47.	Parietal region	HOMO	ND	ND	++	++	Melanoma

LOH=loss of heterozygosity; RER+ = replication error positive; HETERO =heterozygous samples; HOMO= homozygous samples; ND = not determined; - = no expression; + = weak expression, ++ = moderate expression, +++ = strong expression; M = membranous; C= cytoplasmic; N = nuclear localization; PR = primary tumor expression levels

Twenty-four metastases originated from primary lung carcinomas, 9 from breast carcinomas, 5 from colon carcinomas, 2 from gastric, 2 from renal, 1 from sebaceous carcinoma, 1 from neuroendocrine carcinoma, 1 from melanoma, and in two patients with brain metastases primary tumors could not be assessed. When distributing total E-cadherin's changes to specific primaries from which the metastasis originated, primary lung cancers demonstrated changes in: 83.3% of adenocarcinoma; 75% of small cell lung cancer (SCLC); 25% of squamous cell carcinoma; and 12.5% of large cell carcinoma. In comparison to other lung cancer pathologies, the diagnoses adenocarcinoma and SCLC were significantly associated to E-cadherin genetic changes with $\chi 2= 10.364$; df=1; P=0.001.

Metastases from ductal invasive breast carcinoma and those originating from colon

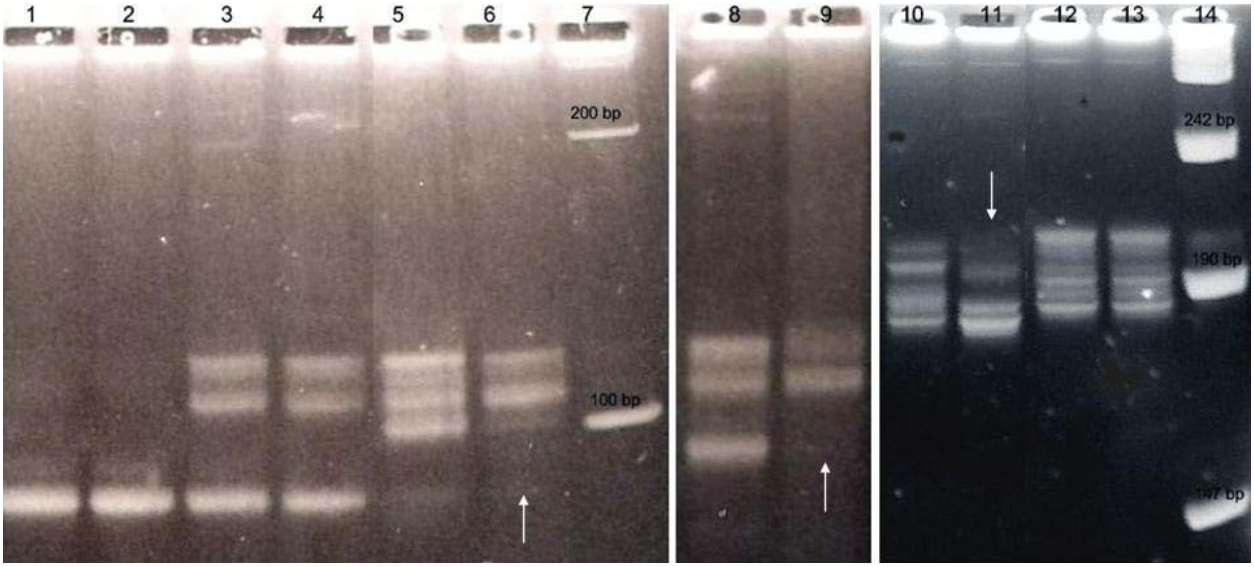

Figure 1. Genomic instabilities, LOH and RER+, of the E-cadherin gene in brain metastases, Spreadex gels (Elchrom Scientific) stained with Sybergold (Molecular Probes). Polymorphic markers D16S265 and D16S398 are shown. Lane 7- 100 bp standard; lane 14- pUC19 Hpa II standard; lanes 1, 3, 5, 8, 10, 12 –corresponding blood samples; lanes 6, 9 - LOHs of the E-cadherin gene (D16S265) indicated by arrows; lane 11 - RER+ sample (D16S398) indicated by arrow; lanes 4, 13- heterozygous patients, lane 2- homozygous patient.

adenocarcinoma harbored LOH in 55.6% and 50% of cases, respectively. One LOH was found each in clear cell renal carcinoma, sebaceous carcinoma and neuroendocrine carcinoma. The metastasis sample analyzed, the origin of the lesions and the detailed histopathological diagnosis are given in Table 2.

In order to determine whether E-cadherin's changes at the genetic level have consequences at the protein level, the expression of E-cadherin proteins was investigated in metastases tissues. Normal levels of E-cadherin staining were scored as +++, and the protein was located along the cell membrane or inside the cytoplasm. Immunostaining showed that overall 83% of samples had weak or moderate E-cadherin expression. Among those downregulated intense downregulation of E-cadherin was noticed in 53.2% of cases. Sixteen out of 19 samples with CDH1's LOH were accompanied with the downregulation of its protein expression (84.2%) as shown in Table 2. Beta-catenin was upregulated in 53.2%, while only 27.7% of our total metastasis sample showed nuclear localization of the protein. In the majority of cases beta-catenin protein was weakly expressed equally to the measurements of normal levels of beta-catenin's staining scored as +.

Our next step was to detect whether the expression and cellular localization of the main downstream wnt signaling effector molecule, beta-catenin, correlated with the changes of E-cadherin gene. We noticed that 21/47 44.7% of samples with lower E-cadherin expression had higher beta-catenin expression than observed in normal tissues. We also noticed that only 4 out of 19 samples showing E-cadherin's LOH (21.1%) had beta-catenin in the nucleus. There was no statistically relevant association between decreased expression of E-cadherin and beta-catenin transfer to the nucleus (Pearson correlation= - 0.047; P=0.758).

Only 4 primary tumors were available for the IHC analysis. Expression patterns in two primary tumors did not differ to their metastatic tissues, while in two primaries from lung cancer higher levels of E-cadherin, and lower levels of beta-catenin than in the corresponding metastases were observed.

The findings on the protein levels are also shown in Table 2. Immunostaining of E-cadherin proteins is demonstrated in Figure 2A and B, and beta-catenin's location in the cytoplasm in Figure 2C. The obtained results were then evaluated by image analysis as staining density, i.e. light permeability (LP). Density was depicted as the intensity of light retained by tissue or tissue transparency, and it is reversely proportional to the protein quantities. The results of immunostaining obtained by three independent observers and by image analysis were compatible and the correlation was very strong at the P=0.0001 level (Pearson's correlation = -0.597). When we correlated our molecular findings with the demographic variables we were unable to demonstrate that changes were significantly associated with the analyzed age groups or sex. Also, the expression levels of the two proteins investigated were not associated to any particular pathohistological diagnosis.

The measured quantity of beta-catenin protein and its localization were not correlated to E-cadherin's genetic changes. We were also unable to establish statistically relevant association between the quantities of beta-catenin obtained by image analysis and its localization, but noticed a weak trend of lower beta-

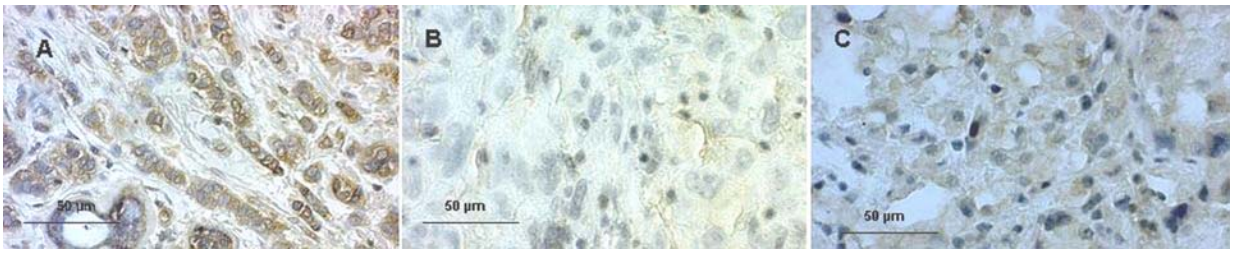

Figure 2. Brain metastasis samples immunohistochemically stained for the expression of E-cadherin and beta-catenin proteins. A. Metastasis tissue demonstrating strong expression of E-cadherin protein. B. Metastasis tissue demonstrating decreased expression of E-cadherin protein. C. Metastasis tissue demonstrating moderate cytoplasmic expression of beta-catenin.

catenin's protein nuclear levels to its levels when it was found in the cytoplasm. On the other hand, we established a statistically relevant correlation between the densitometry measurements of the two expressed proteins. The levels of the two proteins were significantly positively correlated in our total tumor sample at P = 0.008 (Pearson's correlation = 0.381) - when there is high quantity of E-cadherin it is accompanied with the high quantity of beta-catenin, and vice versa, indicating that the quantity of expressed E-cadherin was associated to the quantity of beta-catenin.

5. DISCUSSION

The formation of mobile cells with metastatic potential is the result of multiple consecutive genetic changes that accumulate and represent a critical factor in tumor progression. Genetic profile of human brain metastases is still very much undiscovered and inadequately explained. Therefore, identification of new genes that will improve understanding of the basis of tumor progression and metastasis is very important. With this in mind, the result of this study demonstrates that molecular changes of E-cadherin are frequent among brain metastases. The observed frequency of 42.2%, led us to conclude that gross deletion of the CDH1 gene are an important event in the mechanisms of metastasis formation. Moreover, the functional consequences of the changes were confirmed at the protein level. To our knowledge this is the first report on LOH of the CDH1 gene in brain metastasis tissue.

The primary tumors that metastase to the brain are primarily lung cancer, followed by breast cancer, melanoma, renal cancer and colon cancer (23). These primaries are consistent with the proportions of metastases' origins in our sample. Although, the two main sources of brain metastasis - adenocarcinomas of the lung or the breast - represent different models of the course of the disease (15), our results showed that E-cadherin changes were frequent in metastases from both those malignancies.

In spite of small number of cases in specific subgroup according to primary tumor, the correlation of CDH1 genetic changes to specific lung primary was significant. Metastases originating from lung adenocarcinoma and SCLC were significantly associated to E-cadherin genetic changes (P=0.001). These data are very interesting in light of today's knowledge that the most common sources of brain metastases in adults are especially small cell lung cancer and adenocarcinoma of the lung (2). Primary tumor status appears to be one of the most consistent significant prognostic factors according to the study by Lagerwaard *et al.* (24). It is believed that metastasis must at least in part rely on genetic changes present in the majority of cells which constitute the primary tumor (17). We are aware that some of our subsets according to primary origin consist of small number of tumors and conclusions should be made with caution.

Changes of CDH1 in metastases from breast and colon were also frequent in our analyzed sample. Loss of heterozygosity and reduced membranous expression of E-cadherin in 50% of the ductal breast carcinoma was reported by Sarrio *et al.* (13). E-cadherin changes in colon cancer are also frequently found.

Why does the brain provide a favorable environment for lung or breast cancer cell is a very interesting question. Reports indicate that specific set of genes mediate metastasis to the brain according to the discrete changes. On the other hand, metastasis genes fulfill functions specialized for the microenvironment of the metastatic site and some of them may not necessarily be selected for in primary tumors.

The suppression of E-cadherin expression is regarded as one of the main molecular events responsible for dysfunction in cell-cell adhesion (5). Most tumors have abnormal cellular architecture, and the loss of E-cadherin is a well known prerequisite for tumor cell invasion. It is also important to discus the epithalial-to-mesenchimal transition (EMT) in cancer. This process is similar to developmental events but with the important difference that it is uncontrolled. Epithelial-mesenchymal conversion is an important mechanism for the initial step of metastasis and the hallmark of EMT is the loss of E-cadherin expression. Another hallmark of EMT is the increased expression of intermediate filament proteins such as vimentins. A specific phenomenon has been known to occur in epithelial-mesenchymal transition, the so called cadherin switch where E-cadherin is replaced by N-cadherin (25). This occurrence in tumors is regarded as a sign of invasive behavior and progression. We may speculate that the reduction or the loss of E-cadherin protein is the driving force for metastasis formation and specific molecular interactions at the tumor-brain interface. Prudkin *et al.* (25) demonstrated that primary adenocarcinoma and squamous

cell carcinoma of the lung had high levels of the epithelial-to-mesenchymal transition phenotype, characterized among other things by reduced expression of E-cadherin. In the corresponding brain metastases they noted reversed mesenchymal-to-epithelial transition. The study by Bukholm *et al.* (26) examined the expression of E-cadherin and beta-catenin in lymph node metastases from lobular breast cancers and found the re-expression of both proteins in metastases they examined as if the adherens junctions were functionally reconstituted in metastatic deposits.

Our study on brain metastases did not show such features. The majority of metastases had downregulation of E-cadherin expression with intense downregulation in 53.2% of cases. Similar results were reported by McDonald *et al.* (27) who also detected lower E-cadherin expression in the metastatic lesions. Moreover, Saad *et al.* (28) demonstrated that loss of E-cadherin in patients with adenocarcinoma and squamous cell carcinoma of the lung is significantly associated to the increased risk of developing brain metastases. The results of other authors investigating E-cadherin involvement in brain metastasis (18, 25, 29) collectively demonstrate that E-cadherin is constantly expressed in metastatic deposits. E-cadherin was expressed in the majority of our sample too, but contrary to above studies, different levels of this expression were observed. Arnold *et al.* (18) demonstrated that E-cadherin's expression did not differ between primary lung cancer and brain metastasis pairs in 71% of patients, whereas contrary to our results, expression was greater in the brain metastasis in 29% of patients.

However, biologic spectrum of metastases is wide, heterogenic and difficult to predict, resulting in poor prognosis (30). Their proliferative activity and invasion are important characteristics that should be considered in diagnostics and prognosis. Why is the metastasis process from the lung so fast, while from the breast and the other primaries slower? The velocity of metastasis establishment differs depending on the primary site and may therefore differ according to the genes involved. However, relevant information on genes that are crucial in the metastatic process is not yet used for diagnostic, prognostic or therapeutic purposes (1, 2). The four RER+ phenotypes that we detected indicate that metastatic potential of CDH1 gene might also be connected to microsatellite instability and mismatch repair in specific cases.

Although beta-catenin is frequently mutated in many tumors (31, 32), the results of our analysis did not detect mutations of beta-catenin suggesting that mutations in exon 3 of the CTNNB1 gene are not associated to metastatic process to the brain. We targeted exon 3 of CTNNB1 gene, since it has been reported as mutational hot spot. Reports indicate that mutational activation of the beta-catenin gene is very rare in lung cancer (33) which is consistent to findings in our sample in which great many metastases originated from lung cancer. Moreover, Papay *et al.* (34) associated reduced production of beta-catenin to metastatic potential of primary lung tumors.

Our results demonstrate that relatively low number of samples harboring E-cadherin's gross deletions had beta-catenin located in the nucleus. Beta-catenin's expression and localization were not statistically correlated to the genetic changes of E-cadherin. Furthermore, there was no statistically relevant association between decreased expression of E-cadherin and beta-catenin transfer to the nucleus. These findings are different from our previous work on meningiomas where a significant association between the genetic changes of E-cadherin and the nuclear localization of beta-catenin protein ($P<0.022$) was observed (10). Our results on beta-catenin's distribution regarding cellular localization are also different from our previous work on meningiomas and neuroepithelial brain tumors where nuclear beta-catenin's localization was observed in 46% and 59.4% of tumors, respectively. Immunolocalization of beta-catenin in the nucleus was reported in human malignant tumors by many authors (4) and nuclear location is an indicator of beta-catenin's acquisition of oncogenic activity and the activation of canonical wnt signaling. The amounts of beta-catenin observed in the present study, and the fact that it was not frequently located in the nucleus may indicate that metastasis formation is rather connected to the loss of tissue architecture mediated by E-cadherin and beta-catenin in adherens junctions and not to the activation of wnt signaling.

Based on our previous studies (10, 35) we hypothesized that the loss of E-cadherin protein would indirectly activate wnt signaling, and therefore the levels of the effector beta-catenin would rise in metastases where losses or low quantities of E-cadherin were observed. Interestingly, these assumptions proved not to be true in metastases we investigated. Image analysis showed that the quantities of both proteins were significantly positively correlated ($P = 0.008$) - when there is high quantity of E-cadherin it is accompanied with the high quantity of beta-catenin, and vice versa. The predominantly cytoplasmic localization of beta-catenin and the positive correlation between the quantities of the two proteins may suggest that they probably co-localize in cellular compartment.

Our findings contribute to better understanding of brain metastasis genetic profile and mechanisms of tumor dissemina-tion offering potential diagnostic tools.

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