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HMGA1 protein expression in familial breast carcinoma patients

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ABSTRACT

HMGA protein overexpression is associated with a highly malignant phenotype and it is also causally related to neoplastic cell transformation. Our previous results have shown that HMGA1 was not expressed in normal breast tissue whereas HMGA1 staining was intense in 25% of hyperplastic lesions with cellular atypia and in 60% of sporadic ductal carcinomas. Moreover, HMGA1 protein levels were significantly correlated with c-Erb-B2 expression. These results suggested HMGA1 expression as a novel prognostic factor in breast ductal carcinomas.

In order to investigate whether the HMGA1 detection might have a prognostic role also for inherited breast carcinomas we have analysed the expression of the HMGA1 proteins in 116 breast familial carcinomas associated with *BRCA1* or *BRCA2* or negative for mutations in both genes (BRCAX). HMGA1 expression was weakly positive in 23 (20%), moderately positive in 34 (29%) and strongly positive in 20 (17%) breast carcinomas, and was not detected in 39 of them (34%). Statistical analysis of the immunostaining data showed that HMGA1 was significantly overexpressed, with a more intense staining, in *BRCA2* ($p = 0.0009$) and BRCAX ($p = 0.0134$) patients compared to *BRCA1* ones.

Furthermore, in *BRCA2* positive patients, the expression of HMGA1 seems to correlate with a favourable prognosis with a median overall survival of 65 months and a 5-year survival rate of 80% for HMGA1-negative patients, while median overall survival in the HMGA1-positive subsets was not reached with 5-year survival rates ranging from 84% to 100% of patients ($p = 0.0198$). Conversely, no correlation was found between HMGA1 expression and overall survival in patients carrying inherited mutations in the *BRCA1* and in BRCAX patients.

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1. Introduction

Breast cancer represents the leading cause of morbidity and mortality in women throughout much of the developed world.[1] It accounts for 22% of all female cancer and the estimated annual incidence of breast cancer worldwide is about one million cases. At least 10% of these tumours develop in families with strong aggregations of both breast or ovarian

Table 1 – Correlations between HMGA1 staining score and baseline characteristics of patients.

	No.	%	HMGA1 staining score				p
			0	+	++	+++	
Age, years							
Median	47		49	42	42	45	
Range	23–76		33–76	28–74	23–71	25–67	0.492
Surgery							
Lumpectomy	69	59.5	20	10	25	14	
Mastectomy	43	37.1	17	12	8	6	0.0818
Unknown	4	3.4					
Histology							
Ductal	89	76.7	31	17	25	16	
Lobular	8	6.9	2	2	1	3	
Mixed (ductal + lobular)	9	7.7	3	1	4	1	
Others	10	8.6	3	3	4	–	0.6355
HER2							
Negative (0/1+)	63	54.3	22	15	19	7	
Positive (2+/3+)	1	0.9	–	–	1	–	N.A.
Not done	52	44.8					
Histology grade							
I/II	62	53.4	17	12	21	12	
III	46	39.6	18	9	12	7	0.5937
Unknown	8	6.9					
ER							
Negative	44	37.9	20	11	8	5	
Positive	67	57.7	18	11	24	14	0.0472
Unknown	5	4.3					
PR							
Negative	56	48.2	24	12	13	7	
Positive	55	47.4	14	10	19	12	0.1547
Unknown	5	4.3					
Contralateral breast cancer							
Yes	32	27.6	10	6	10	6	
No	81	69.8	28	16	23	14	0.9809
Unknown	3	2.6					
Second primary cancers							
Ovarian cancer	7	6.0	4	2	1	–	
Basal cell carcinoma	1	0.9	1	–	–	–	
Cervix carcinoma	1	0.9	–	–	1	–	
Melanoma	1	0.9	1	–	–	–	
CML**	1	0.9	–	–	1	–	
Larynx carcinoma	1	0.9	–	–	–	1	
Malignant phylloid tumour	1	0.9	–	–	–	1	
Colon carcinoma	1	0.9	–	–	1	–	
Renal carcinoma	1	0.9	–	1	–	–	
Parotid tumour	1	0.9	–	–	1	–	NA*
Familial aggregations							
Breast cancers	88	75.9	29	16	26	17	
Breast/ovarian cancers	26	22.4	10	6	7	3	0.7609
Unknown	2	1.7					
Association of DCIS							
Present	21	18.1	10	5	2	4	
Not present	95	81.9	29	18	32	16	0.1595

NA*: not applicable; CML**: chronic myeloid leukaemia.

cancers showing an autosomal dominant transmission pattern.[1] *BRCA1* and *BRCA2* mutations are responsible for 15–20% of site-specific breast cancer families and the majority of breast and ovarian cancer families.[2,3] Hereditary breast carcinomas occurring in *BRCA1* patients have distinct histopathologic and immunophenotypic features. In fact, they generally show a higher grade, a pushing margin growth pattern, negativity for oestrogen receptor (ER), progesterone receptor (PR) and Erb-B2 expression, and a high proportion of lymphocytic infiltration compared with sporadic and familial non-*BRCA1/2* breast cancer, and are frequently associated with a poor prognosis.[4] Conversely, *BRCA2* tumours are not clearly associated with a specific subtype, but invasive lobular, pleomorphic lobular, tubular and cribriform forms have been reported more frequently in this group.[4–6] However, even though there are some evidences of an association between poor prognostic factors and *BRCA1/2* mutations, it is still a matter of discussion whether the prognosis of familial breast cancer differs from that of sporadic cases. The High Mobility Group A (HMGA) family comprises four proteins: HMGA1a, HMGA1b, HMGA1c and HMGA2 (formerly HMGI, HMGY, HMGI/R and HMGI-C, respectively). They are encoded by two distinct genes, the HMGA1 proteins being products of the same gene generated through alternative splicing.[7,8] By interacting with the transcription machinery, HMGA1 proteins alter the chromatin structure and, thereby, regulate the transcriptional activity of several genes.[9] They seem to play their major physiological role during embryonic development.[10] HMGA protein expression has been found abundant in several malignant neoplasias, including pancreas,[11] thyroid,[12] colon,[13] breast,[14] lung,[15] ovary,[16] prostate carcinomas,[17] squamous carcinomas of the oral cavity[18] and head and neck tumours.[19] HMGA overexpression is mainly associated with a highly malignant phenotype, also representing a poor prognostic index since HMGA overexpression often correlates with the presence of metastasis, and with a reduced survival.[15,20] HMGA overexpression has a critical role in the process of carcinogenesis and is not merely a result of cell transformation. In fact, the block of HMGA proteins' synthesis prevents rat thyroid cell transformation by acute murine retroviruses,[21] and HMGA overexpression induces rat and mouse fibroblast transformation.[22]

We have previously shown that HMGA1 was not expressed in normal breast tissue whereas HMGA1 staining was intense in 25% of hyperplastic lesions with cellular atypia and in 60% of sporadic ductal carcinomas, and weak in fibroadenomas and in hyperplastic lesions without cellular atypia. Moreover, HMGA1 protein levels were significantly correlated with c-Erb-B2 expression, but not with histological grade.[14] It has also been demonstrated by our group that HMGA1b protein binds to and inhibits the activity of both human and mouse *BRCA1* promoters and the presence of an inverse correlation between HMGA1 and BRCA1 expression in human breast carcinoma cell lines and tissues.[23]

The aim of our study was to investigate whether HMGA1 protein expression might be a prognostic indicator in *BRCA1/2* and BRCAX-associated breast carcinomas. Here we report the analysis by immunohistochemistry of the expression of the HMGA1 proteins in 116 familial breast carcinomas associated with *BRCA1* or *BRCA2* or negative for mutations in both genes (BRCAX). Interestingly, HMGA1 expression seems to correlate with a longer survival time in *BRCA2* positive patients. Conversely, there is no correlation between HMGA1 expression and overall survival in patients carrying inherited mutations in the *BRCA1* gene and in BRCAX cases.

2. Materials and methods

2.1. Patients

The cases included in the study were 116 patients affected by breast familial carcinomas seen at the Istituto Nazionale Tumori (INT) of Milan from 1990 to 2002. Enrolled cases were

Table 2 – Associations between *BRCA* status and HMGA1 staining scores.

BRCA germ-line mutations	HMGA1 staining score*, N′ (%)				p″
	o	+	++	+++	
BRCA1 (N = 31)	16 (51.6)	8 (25.8)	7 (22.6)	0 (0.0)	
BRCA2 (N = 23)	4 (17.4)	6 (26.1)	4 (17.4)	9 (39.1)	
BRCAX (N = 62)	19 (30.6)	9 (14.5)	23 (37.1)	11 (17.8)	0.0015

N′, number of cases.
p″, p-value.
* The percentage of cells with nuclear staining for HMGA1 was scored from 0 to 3: 0, <10% of positive cells; 1+, 11–40% of positive cells; 2+, 41–70% of positive cells; 3+, 71–100% of positive cells.

Table 3 – Two variables comparisons of BRCA status according to HMGA1 scores.

Comparison of BRCA status	p
BRCA1 versus *BRCA2*	0.0009
BRCA1 versus BRCAX	0.0134
BRCA2 versus BRCAX	0.0532

from families with a history of breast and/or ovarian cancer complying with the intake criteria to BRCA testing in use at INT.[24] All subjects received genetic counselling and provided a written consent to mutation testing and for the use of their biological samples for research purposes, approved by the local ethical committee in accordance with Italian current regulations. Standard therapeutic strategies were applied for these patients and none received neo-adjuvant treatments. The presence of constitutional mutation of the BRCA genes were verified by screening of all coding exons and splice sites by direct sequencing or denaturing high performance liquid chromatography (DHPLC), according to

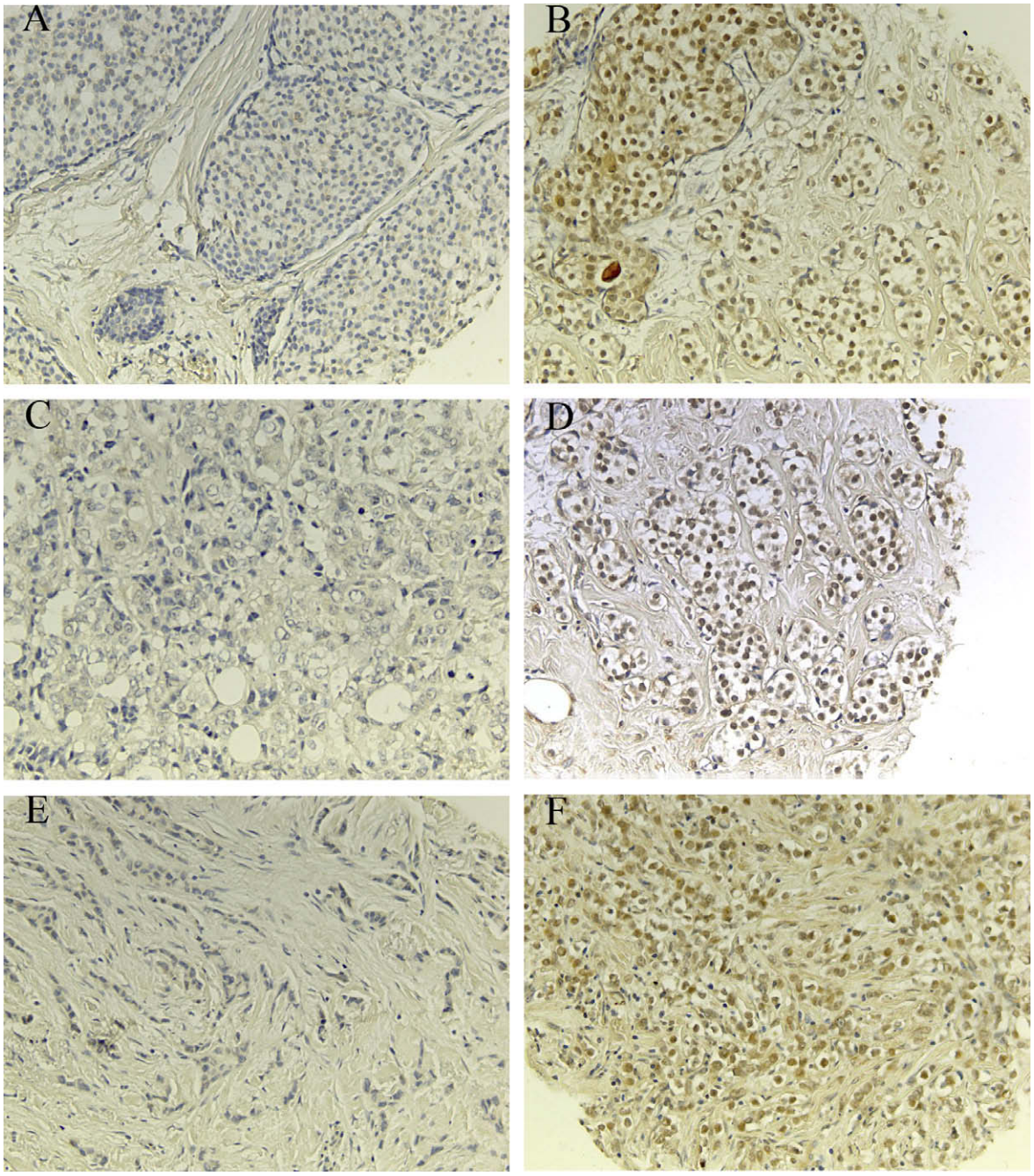

Fig. 1 – Immunohistochemical analysis of HMGA1 protein expression in *BRCA1* and *BRCA2* and BRCAX-related breast tumour on a tissue microarray. Paraffin sections from breast cancer carrying germ-line mutations in *BRCA* genes were analysed by immunohistochemistry using antibodies raised against a specific HMGA1 peptide. Panel A: Immunostaining of a *BRCA1* breast carcinoma (200×), showing absent HMGA1 protein expression. Panel B: Immunostaining of a *BRCA1* breast carcinoma (200×), showing nuclear immunoreactivity. Panel C: Immunostaining of a *BRCA2* breast carcinoma (200×) negative for HMGA1 protein expression. Panel D: Immunostaining of a *BRCA2* breast carcinoma (200×). The immunoreactivity is present in the nucleus of malignant cells. Panel E: Immunostaining of a non-*BRCA1/2* breast carcinoma (200×) showing no HMGA1 protein expression. Panel F: Immunostaining of a non-*BRCA1/2* breast carcinoma (200×) showing nuclear immunoreactivity.

published protocols[25,26] with minor modifications. Tumours from carriers of *BRCA1* or *BRCA2* allelic variants of uncertain clinical significance were not included. Clinical and histopathological data were obtained from medical records. In cases where the information on ER, PR and HER2 status was not already available, this was assessed as previously described.[27]

2.2. Tumour samples and tissue microarray (TMA) construction

All tumours were invasive carcinomas and were classified for type and grading by reviewing haematoxylin–eosin slides by the pathologist, blinded to *BRCA1* and *BRCA2* germ-line mutational status. Representative tumour areas were carefully selected on haematoxylin–eosin-stained sections and marked on individual paraffin blocks. Two tissue cores (0.6-mm diameter) were obtained from each specimen. In addition, nine non-neoplastic breast tissue samples were included as controls. The tissue cores were precisely arrayed into a new paraffin block using a TMA workstation. A haematoxylin–eosin-stained section was reviewed to confirm the presence of morphologically representative areas of the original lesions.

2.3. Immunohistochemistry of BRCA1 and BRCA2 and BRCAX-associated tumours

For immunohistochemistry, 2 μm paraffin sections from the TMA were deparaffinised and then placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. The slides were then incubated overnight at 4 °C in a humidified chamber with the antibodies diluted 1:100 in PBS. The slides were subsequently incubated with biotinylated goat anti-rabbit IgG for 20 min (Vectostain ABC kits, Vector Laboratories) and then with premixed reagent ABC (Vector) for 20 min. The immunostaining was performed by incubating the slides in diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS, pH 7.6, for 5 min, and after chromogen development, the slides were washed, dehydrated with alcohol and xylene and mounted with coverslips using a permanent mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system. The antibodies used in this study were raised against the synthetic peptide SSSKQQPLASKQ specific for the HMGA1 protein.[28] They were affinity purified against the synthetic peptide. Tissue samples were scored as positive for immunohistochemistry when tissue immunoreactivity was detected in at least 10% of the cells. As expected, immunohistochemical reactivity was predominantly localised to the cells nuclei. Negative controls were performed by omitting the first antibody. The specificity of the reaction was confirmed by the lack of tissue immunoreactivity after pre-incubation of the antibody with molar excess of the HMGA1 synthetic peptide.

2.4. Statistical analysis

Correlations between HMGA1 expression, baseline patient features and *BRCA* status were studied by contingency tables and the χ^2 test. Overall survival (OS) was defined as the time elapsed from the date of the initial diagnosis to death or to the date of the last available information on vital status. Kaplan–Meier product limit method was applied to draw OS curves. Univariate analysis was done with the log-rank test.

3. Results

Of the 116 patients affected by familial breast carcinoma entered in this study, 31 carried *BRCA1* mutations, 23 had *BRCA2* mutations, and 62 patients did not show any mutation in both these genes (BRCAX). Clinical–pathological data of the analysed patients are shown in Table 1. Median age of patients was 47 years (range: 23–76). Sixty-nine patients (59.5%) underwent lumpectomy, 43 (37.1%) underwent mastectomy. The histotype was ductal in 89 (76.7%) patients, lobular in 8 (6.9%). HER2 was positive (2+/3+) in 1 patient (0.9%). Sixty-two patients had grade I/II tumours (53.4%), while 46 (39.6%) were classified of grade III. Oestrogen and progesterone receptors were negative in 44 (37.9%) and 56 (48.2%) breast cancers, respectively. In 32 patients (27.6%) there was a controlateral breast cancer. The predominant second primary cancer was ovarian cancer. There was a familial aggregation of breast cancers in 88 patients (75.9%) and of breast/ovarian cancers in 26 patients (22.4%). Association with an *in situ* ductal carcinoma was present in 21 cases (18.1%).

The breast carcinoma samples and nine samples deriving from normal breast tissue were analysed for the HMGA1 expression by immunohistochemistry using specific antibod-

Table 4 – Univariate analysis of prognostic factors for survival.

Variable	Events/patients	Five-year survival rate	Median survival	*p*
Age (<65 versus >65 years)	20/106; 3/10	89% versus 63%	N.R. versus N.R.	*0.1104*
Tumour grade (I/II versus III)	9/61; 13/47	90% versus 81%	N.R. versus N.R. versus N.R.	*0.1461*
Mastectomy versus lumpectomy	10/43; 12/71	84% versus 86%	N.R. versus N.R. versus N.R.	*0.7364*
HMGA1 staining scores (0 versus 1 versus 2 versus 3)	11/38; 4/21; 6/32; 2/20	100% versus 84% versus 78% versus 89%	N.R. versus N.R. versus N.R. versus N.R.	*0.7122*
BRCA1 versus *BRCA2* versus BRCAX	11/31; 4/24; 8/61	72% versus 92% versus 94%	156 months versus N.R. versus N.R.	*0.0431*
ER–versus ER+	13/46; 9/64	77% versus 94%	N.R. versus N.R.	*0.0703*

N.R.: not reached.
ER: oestrogen receptor.

ies raised versus the N-terminal region of the HMGA1 protein following the already described procedure.[14] The results of this analysis are reported in Table 2.

While none of the normal breast tissues showed HMGA1 staining, HMGA1 expression was moderately positive (+) in 23 (19.8%), clearly (++) and strongly positive (+++) in 54 carcinomas (46.5%), whereas it was not detected in 39 (33.6%) tumour samples. The frequency of *BRCA1*, *2* and BRCAX was associated with HMGA1 expression ($p = 0.0015$) as shown in Table 2. HMGA1 overexpression was found more frequently, and also with a more intense average staining, in *BRCA2* ($p = 0.0009$) and BRCAX ($p = 0.0134$) breast carcinomas in comparison with the *BRCA1* ones (Table 3). In fact, 56.5% of the carcinoma samples from patients carrying *BRCA2* mutations and 54.9% from BRCAX patients mutations showed a positive staining evaluated as 2+ or 3+, while only 22.6% of the *BRCA1* patients showed high HMGA1 (2+/3+) expression levels. In Fig. 1 we show some representative immunohistochemical analyses: an intense nuclear staining was clearly observed in *BRCA1*-, *BRCA2*- and BRCAX-associated breast carcinomas (Fig. 1B, D, F), whereas in Fig. 1A, C, E we show some representative cases of a *BRCA1*-, *BRCA2*- and BRCAX-associated breast carcinomas which do not express HMGA1 protein.

At the time of this analysis, after a median follow-up for alive patients of 60.9 months, 23 patients died. Univariate analysis of prognostic factors for overall survival (OS) is summarised in Table 4. Median OS was 54.0 months. *BRCA1* gene mutation had a significant prognostic value for OS at univariate analysis ($p = 0.0431$). The 5-year OS rate was 72% for patients with *BRCA1* mutations, 94% for BRCAX patients and 92% for patients with *BRCA2* mutations. The graphic pattern of Kaplan–Meier estimated curves according to *BRCA* status is shown in Fig. 2. Multivariate analysis was not done because of low number of events. The prognostic role of HMGA1 proteins was studied in *BRCA* subsets. HMGA1 expression did not show a prognostic significance in patients carrying inherited mutations in the *BRCA1* gene and in BRCAX patients (Fig. 3A and C). Conversely, *BRCA2* patients expressing HMGA1 proteins showed a longer survival time in comparison with the *BRCA2* patient group with negative or low expression of HMGA1 (Fig. 3B). For *BRCA2* HMGA1-negative tumours median OS was 65 months while for *BRCA2* HMGA1-positive tumours it was not reached ($p = 0.0198$).

To exclude that the correlation between HMGA1 expression and prognosis might reflect oestrogen receptor (ER) status rather than HMGA1 expression we have analysed the

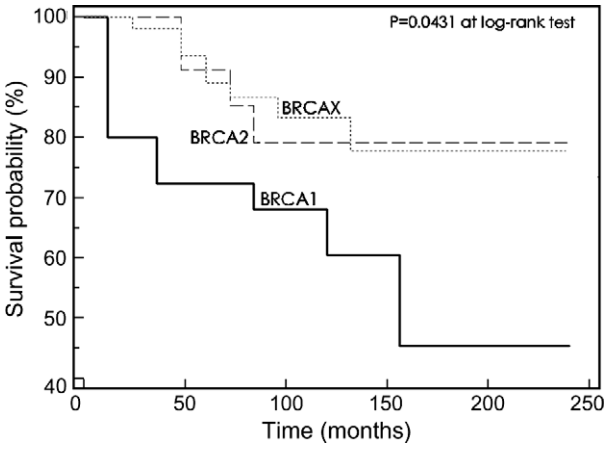

Fig. 2 – Kaplan–Meier for all patients according to *BRCA* status. BRCA1 status was associated with an unfavourable prognosis ($p = 0.0431$).

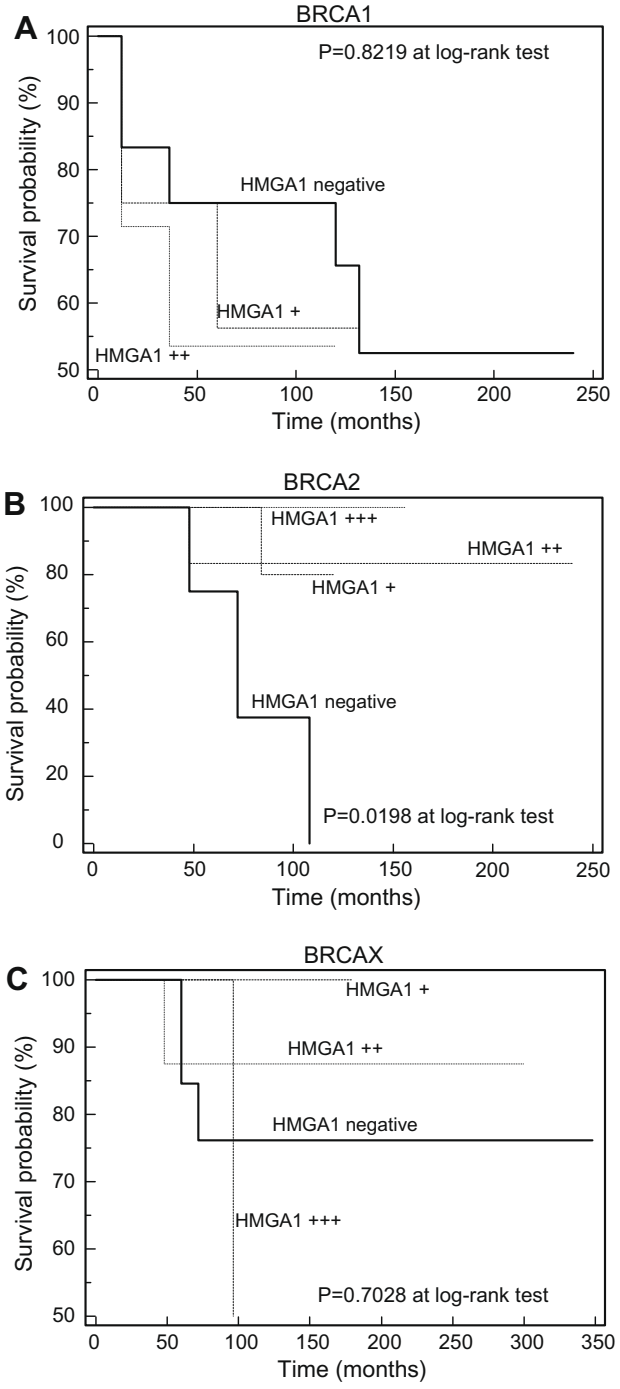

Fig. 3 – Kaplan–Meier survival curves in BRCA subsets (A, BRCA1; B, BRCA2 and C, BRCAX) according to HMGA1 expression. In BRCA2 patients the expression of HMGA1 was associated with a favourable prognosis ($p = 0.0198$).

association between ER and HMGA1 expression according to the *BRCA* status (Supplementary Table 1bis). No association was found between ER status and HMGA1 in *BRCA2* patients. Therefore, the good prognosis in *BRCA2* patients is likely due to the HMGA1 overexpression.

4. Discussion

HMGA1 expression correlates with the malignant phenotype in several cancer histotypes and its abundance is frequently associated with a poor prognosis. Our previous study has shown that 60% of ductal carcinomas were positive for HMGA1 immunostaining, and that HMGA1 expression significantly correlated with that of c-Erb-B2.

In this study we have analysed for HMGA1 expression 116 familial breast carcinoma samples, including 31 and 23 cases positive for *BRCA1* and *BRCA2* germ-line mutations, respectively, and 62 samples negative for mutations in both these genes (BRCAX). Seventy-seven cases showed a positive immunostaining. This percentage was not so different from that previously reported by us on sporadic cases. However, HMGA1 overexpression was found more frequently, and also with a more intense average staining, in *BRCA2* and BRCAX breast carcinomas in comparison with the *BRCA1* ones. Moreover, some interesting considerations come from the analysis of the single groups. In fact, while HMGA1 expression does not correlate with the prognosis of the patient group carrying mutations in *BRCA1* or negative for mutations in *BRCA1* and *BRCA2*, in the case of the *BRCA2* patient group the HMGA1 expression seems to correlate with a good prognosis with an increased survival compared to that of the patients of the same group but in absence of HMGA1 positivity. These results appears quite surprising since all the previous data analysing cancers of different tissue origin have shown that the carcinoma samples overexpressing the HMGA1 proteins are characterised by a higher stage in comparison to the samples negative for HMGA1 expression. It could be based on the different functions of the HMGA1 protein depending on the cellular context. Indeed, we have previously demonstrated that HMGA1 may behave as oncogene or tumour suppressor gene depending on the cellular context: NK lymphoma and pituitary adenomas appear when the HMGA1 gene is overexpressed,[29] whereas the lack of HMGA1 expression, as it occurs in HMGA1 null mice, is associated with the development of B cell lymphomas.[30] Therefore, we could hypothesise that the impairment of the *BRCA2* function may cause cellular modifications that can account for a protective function of HMGA1. Alternatively, the previously described ability of HMGA1 to downregulate *BRCA1* expression[23] may suggest that the lower expression of HMGA1 in the *BRCA1* group of patients is indicative of being less important for the pathogenesis of breast cancer where *BRCA1* function is impaired by a different mechanism.

In conclusion, our data show that HMGA1 overexpression is less frequent in *BRCA1* patients in comparison to sporadic, *BRCA2* and BRCAX breast carcinoma patients, and does not seem to correlate with a bad prognosis in familial breast carcinoma patients. By contrast, it might even represent a good prognostic factor for the breast cancer patients carrying a mutated *BRCA2* gene.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.10.015.

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