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Pharmacogenetic interaction analysis of *VEGFR-2* and *IL-8* polymorphisms in advanced breast cancer patients treated with paclitaxel and bevacizumab

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Aim: To investigate pharmacogenetic interactions among *VEGF-A*, *VEGFR-2*, *IL-8*, *HIF-1α*, *EPAS-1* and *TSP-1* SNPs and their role on progression-free survival in a population of metastatic breast cancer patients treated with bevacizumab in combination with first-line paclitaxel. **Patients & methods:** Analyses were performed on germline DNA obtained from blood samples and SNPs were investigated by real-time polymerase chain reaction technique. The multifactor dimensionality reduction methodology was applied to investigate the interaction between SNPs. **Results:** One hundred and thirteen patients were enrolled from eight Italian Oncology Units (clinicaltrial.gov: NCT01935102). The multifactor dimensionality reduction software provided two pharmacogenetic interaction profiles consisting of the combination between specific *VEGFR-2* rs11133360 and *IL-8* rs4073 genotypes. The median progression-free survival was 14.1 months (95% CI: 11.4–16.8) and 10.2 months (95% CI: 8.8–11.5) for the favorable and the unfavorable genetic profile, respectively (HR: 0.44, 95% CI: 0.29–0.66, $p < 0.0001$). **Conclusion:** The pharmacogenetic statistical interaction between *VEGFR-2* rs11133360 and *IL-8* rs4073 genotypes may identify a population of patients with a better outcome.

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Keywords: advanced breast cancer • angiogenesis • bevacizumab • first-line chemotherapy • multifactor dimensionality reduction methodology • paclitaxel • pharmacogenetics • single nucleotide polymorphisms

The humanized monoclonal antibody bevacizumab is currently approved in combination with standard chemotherapy for the treatment of several cancers [1–4]. Three comparative studies assessed the role of bevacizumab combined with first-line chemotherapy in HER2-negative metastatic breast cancer (MBC) patients, showing a significant improvement in progression-free survival (PFS) of the combination as compared with chemotherapy alone [3,5,6]. The hazard ratios (HRs) for the PFS (the primary purpose the studies were designed for) ranged between 0.48 and 0.67 [3,5,6]. Nevertheless, because of the lack of any benefit in terms of overall survival (OS), the US FDA revoked the initial approval of bevacizumab for the first-line treatment of MBC patients [7]. Despite the FDA decision, many argued that when a long survival post first-line progression is expected, such as in breast cancer, the lack of an observed benefit in OS could not mean a lack of improvement in OS for the first-line of treatment [8].

A rational approach to the unresolved issue if bevacizumab could be considered one of the standards of treatment, might be the identification of validated predictive biomarkers for selecting those patients with the best chance of response to bevacizumab. With this approach, a positive correlation between the best PFS and the OS might also be expected. Because the improvement in PFS from bevacizumab seems to be of iden-

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tical magnitude for all subgroups of patients chosen through clinical and pathological characteristics [9], selective biomarkers may be needed. In this research area, many attempts have been done but, despite the efforts, no validated biomarkers are available in the clinical practice [10].

All the isoforms of VEGF-A, the target of bevacizumab and its receptor-2 (VEGFR-2) have been the most investigated as possible markers of bevacizumab response [11]. Biomarkers analyses from several studies conducted on cancer patients suggested a possible prognostic value of both circulating VEGF-A and VEGFR-2, rather than predictive [12]. These data have been confirmed by analyses performed in AVF2107g, AVOREN and AVAil trials [13]. Instead, analyses from AVADO, AViTA and AVAGAST trials have shown (with a novel enzyme linked immunosorbent assay) that higher plasma concentrations of both VEGF-A and VEGFR-2 at baseline appear to correlate with a better PFS in patients treated with bevacizumab as compared with PFS of the population treated with chemotherapy alone [14–16]. The role of VEGF-A baseline in predicting the response to bevacizumab remains a challenge and the prospective MERiDiAN trial is now being performed to answer this question [10].

Several pharmacogenetic studies have evaluated the role of both germline and somatic gene polymorphisms of angiogenic pathways to predict bevacizumab treatment outcome. Although the findings of initial studies were promising, subsequent reports have produced contrasting results [14,17–19]. Due to the retrospective nature of these studies and to their inconclusive results, none of the evaluated SNPs can be considered as predictive markers [20].

Many authors have rightly pointed out the unlikeness that just a SNP can predict the drug response, mainly due to the complexity of the involved biological systems [21–23]. Therefore, the bevacizumab response could depend on many factors. Indeed, the effect of a SNP on their corresponding genes may be the result of an interaction between the polymorphisms of other genes. This phenomenon is defined nonlinear interaction or epistasis [24,25]. Therefore, the current approach of correlating the bevacizumab response to a SNP may be replaced by a genetic analysis of the interaction between SNPs. The aim of this new procedure should be the generation of a genetic profile with a predictive value. Moore *et al.* have created and validated a methodology, called multifactor dimensionality reduction (MDR), to identify a genetic profile with the ability to predict the drug response [26].

On the basis of these hypotheses, we conducted a retrospective study to assess the ability of MDR methodology to identify a pharmacogenetic profile of polymorphisms associated with PFS in an unselected population of MBC patients treated with bevacizumab combined with first-line paclitaxel. The aim was to identify a genetic profile for investigating its possible perspective predictive role of bevacizumab response in MBC patients.

Patients & methods

Study population

One hundred and thirteen patients from eight Italian divisions of Medical Oncology, with histologically confirmed HER2-negative MBC, were treated with a first-line therapy including bevacizumab 10 mg/m^2 iv. on days 1 and 15 combined with first-line paclitaxel 90 mg/m^2 iv. on days 1, 8 and 15, every 4 weeks, from January 2009 until September 2012, and they were assessed for the present pharmacogenetic study. Fifty-six MBC patients treated with a first-line chemotherapy including paclitaxel without bevacizumab was also enrolled to test the role of SNPs eventually related to PFS. Basal and pathological characteristics recorded from both groups were the following: age (≤ or >65 years); Eastern Cooperative Oncology Group (ECOG) performance status (0 or 1–2); hormonal-receptor status (positive or negative); previous adjuvant chemotherapy (none, anthracycline or anthracycline plus taxane); previous hormonal therapy (adjuvant or metastatic); disease-free interval from the first diagnosis of breast cancer (≤ or >12 months); extent of disease (≤ or >3 sites); location of disease (viscera or bone); disease evaluation (measurable or nonmeasurable) and bevacizumab maintenance (yes or no). Patients with HER2-positive, were excluded from the present study.

The treatment with chemotherapy was continued until either disease progression occurred or unacceptable toxicities registered or it was stopped for medical choice. The bevacizumab maintenance was continued and hormone therapy added for both groups when indicated, until disease progression or unacceptable toxicities occurred.

Sites of metastatic disease were radiologically re-evaluated according to the RECIST criteria 1.1 [27], in patients with measurable disease. In patients without measurable lesions, progression of disease was defined when new lesions appeared or when existing lesions evolved. Likewise, in the case of nonmeasurable lesions, deterioration of clinical condition not due to treatment toxicity, was defined as progression of disease.

PFS was defined as the period of time from the beginning of the treatment to the first observation of disease progression as above described, or death from any cause. All patients were assessed for response, PFS and OS. Each patient entering the study signed

the informed consent. The protocol was approved by ethic committee of Azienda Ospedaliera-Universitaria Pisana, Pisa, Italy, (CESM-AOUP 3077/2010; clinicaltrial.gov identifier NCT01935102, for Pisa and Pontedera Hospitals) and by the ethic committees of all participating centers: Comitato Etico dell'Azienda Ospedaliera San Gerardo di Monza, Comitato Etico dell'Azienda Provinciale per i Servizi Sanitari della Provincia Autonoma di Trento, Comitato Etico Locale dell'Azienda USL 3 di Pistoia (for Pistoia and Pescia Hospitals), Comitato Etico Interaziendale dell'Azienda Ospedaliera S. Salvatore di Pesaro, della ASUR Zona Territoriale 1 di Pesaro e Zona Territoriale 2 di Urbino, Comitato Etico Locale dell'Azienda USL 12 di Viareggio.

SNP selection

The SNPs included in our study (Table 1) were selected on the basis of four main considerations: thus far no clear or described association has been reported between the chosen SNPs and prognosis or response to therapy of breast cancers; some of the SNPs have been significantly associated with therapeutic efficacy of antiangiogenic drugs (e.g., bevacizumab or angiokinase inhibitors) in other tumor types; some of SNPs have been associated with modulation of gene expression, or with different plasma levels of the protein; although for some of chosen SNPs the phenotypic effects are still undefined or controversial, possible gene–gene interactions could determine previously undescribed statistical epistatic effects [18,28–34].

Genotyping analyses

Blood samples (3 ml) were collected in EDTA tubes and stored at -80°C. Genes and polymorphisms involved in the angiogenesis pathway and already suggested as predictors of bevacizumab response, were chosen for the present analyses. In the Table 1 the selected polymorphisms are reported. Germline DNA extraction was performed using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Allelic discrimination of genes was performed using an ABI PRISM 7900 SDS (Applied Biosystems, Carlsbad, CA, USA) and with validated TaqMan® SNP genotyping assays (Table 1; Applied Biosystems). PCRs were carried out according to the manufacturer's protocol. Genotyping was not performed until an adequate number of events (>80% on study population) was reported in terms of PFS.

Statistical analysis

The first aim of this retrospective analysis was to evaluate the possible influence of these investigated gene polymorphisms to PFS in an unselected population of MBC patients treated with bevacizumab combined with first-line paclitaxel. The aim was to identify a genetic profile for investigating its possible perspective predictive role of bevacizumab response in MBC patients. The secondary end-points were the correlations with OS and response rate. All polymorphisms were analyzed for deviation from the Hardy–Weinberg Equilibrium (HWE) by means of comparison between observed allelic distributions with those expected from the HWE by on χ^2 test. None of the polymorphisms significantly deviated HWE (see Supplementary Table 1; see online at: www.futuremedicine.com/doi/suppl/10.2217/pgs.14.140).

Any correlation between gene polymorphisms and response rate was analyzed by the two-sided Fisher's Exact Test. The association between each individual polymorphism and the most relevant clinical–pathological characteristics with PFS was tested using a Cox proportional hazards model. In these analyses we used Bonferroni's correction and the p-value <0.00357 (0.05/14 SNPs = 0.00357) was accepted as statistically significant. The MDR methodology was applied (using version 2.0 β 6 of MDR software available on [46]) to investigate the role of an interaction between gene polymorphisms in identifying biomarkers associated with the greater PFS in this population of patients [26]. The genotype combination with the highest PFS benefit related with an OS improvement was chosen for further analyses. The difference in PFS between favorable genetic profiles and the unfavorable genetic profiles were assessed with the log-rank test and the Kaplan–Meier method to evaluate survival curves. A Cox proportional hazards model, with the possible genetic profiles and the clinical and pathological patient characteristics individually related with the PFS, was used to calculate the adjusted HR and the 95% CI. The Kaplan–Meier and Cox proportional hazards analyses were performed using the SPSS version 17.0 (SPSS, Chicago, IL, USA). Also for the genotype combination we used a statistical correction. Indeed, the p-value for the statistical significance was obtained using 1000-fold permutation testing (software available on [47]).

Given an HR, statistically significant with $\alpha = 0.05$, equal to 1.9 (as for the group treated with paclitaxel-bevacizumab), the power of a sample of 56 subjects is equal to 65%. The power of the sample treated with paclitaxel-bevacizumab (HR: 1.9, n = 113) was equal to 91%.

Results

One-hundred and thirteen patients entered the pharmacogenetic analysis. The median number of cycles administered were 7 (range: 3–10) and main-

Table 1. Selected genes and polymorphisms of the angiogenesis pathway that have been chosen for the present analysis.

Genes and rs number	Genetic alteration	Effect of polymorphisms	TaqMan SNP genotyping assays
VEGF-A			
rs699947	A>C	Decreased VEGF production for A allele [35] and increased VEGF expression for A allele [36]	C__8311602_10
rs833061	C>T	Increased promoter activity for T allele [37]	C__1647381_10
rs3025039	C>T	Decreased VEGF levels for the T allele [38,39]	C__16198794_10
rs1570360	G>A	Decreased VEGF production and expression for A allele [35,36]	C__1647379_10
rs699946	A>G	GG carriers have a 27% increased median VEGF expression compared with AG and AA (wild-type) carriers [40]	C__1647395_10
rs2010963	G>C	Decreased VEGF release for C allele [41] and increased serum VEGF for C allele [42]	C__8311614_10
VEGFR-2			
rs2305948	C>T	Val295Ile; protein product may be altered [43]	C__22271999_20
rs11133360	T>C	Undefined [40]	C__26111278_10
rs2071559	A>G	Alteration of the binding site for transcriptional factor E2F [44]	C__15869271_10
rs1870377	T>A	Gln472His; amino acid substitution located in the extracellular ligand binding region [44]	C__11895315_20
HIF-1α			
rs11549465	C>T	Pro582Ser [43]	C__25473074_10
TSP-1			
rs2228262	A>G	Asn700Ser [43]	C__16170900
EPAS-1			
rs4145836	G>A	Undefined [40]	C__32329435_10
IL-8			
rs4073	A>T	A allele increased IL-8 production [45]	C__11748116_10

tenance with bevacizumab alone was continued in 80 patients (74.4%).

All the 113 patients entered the study were evaluated for the response. Fifteen (13%) and 62 patients (55%) experienced a complete and a partial response, respectively, 27 patients (24%) reported a stable disease (SD) and in nine patients (9%) a progression was observed. None of the analyzed polymorphisms affected the response rate.

When the present analysis was performed, 99 of 113 patients (87.6%) progressed and 66 of 113 patients (54.9%) died from the metastatic disease. No patients died of cancer-unrelated causes. After a median follow-up of 25+ months (range: 4.5–69.5+ months), median PFS and median OS were 11.6 months (95% CI: 10.5–12.7 months) and 31.3 months (95% CI: 23.7–38.9 months), respectively. PFS was reached by 87.6% of patients. Data were censored after 70 months.

In Table 2 are reported the associations of clinical and pathological characteristics with both PFS and OS. Hormonal-receptor status confirmed its role in determining the prognosis of this group of patients and, of note, in the group of patients who continued bevacizumab, over the patients who interrupted it at the end of chemotherapy without a evidence of a disease progression, both a greater PFS and OS was observed. In Table 3 are reported the associations of clinical and pathological characteristics with PFS in the group of patients (n = 56) that received paclitaxel without bevacizumab. The main characteristics of these patients were superimposable with the 113 patient study cohort.

The Cox proportional hazards analysis, applied to evaluate the correlation between each single polymorphism with both PFS and OS, did not reveal significant positive association between *VEGFR-2*rs2071559 AG carriers, A-variants of *EPAS-1* rs4145836 and *VEGF-A*rs2010963 CC carriers with PFS (Table 4), after Bonferroni's correction. No significant associations were observed with OS (data not shown).

The MDR program revealed a genetic interaction profile, consisting of the combination between specific *VEGFR-2* rs11133360 and *IL-8* rs4073 genotypes, significantly associated with PFS (Supplementary Figure 1). Particularly, two pharmacogenetic profiles were identified in patients, as reported in Table 5. The first one was associated with a greater PFS benefit whereas the second one with a lower PFS after paclitaxel plus bevacizumab treatment.

The MDR software provided other combinations, including those with more than two genes, which were associated with PFS, but they were not considered further because of a much lower HRs for the PFS and the lack of any OS improvement for the favorable genotypes (data non shown) if compared with the combination reported in Table 5.

The median PFS for the favorable genetic profile was 14.1 months (95% CI: 11.4–16.8 months) versus the 10.2 months of the unfavorable genetic profile (95% CI: 8.8–11.5 months; p < 0.0001, log-rank test; Figure 1A). The Cox proportional hazards model, which was performed to assess the adjusted HR for the PFS of the favorable genetic profile, showed a value of 0.48 (95% CI: 0.32–0.72; p = 0.0004; Table 6). Remarkably, the patients included in the favorable genetic profile also had the best probability of OS benefit, even though the difference was not statistically significant as compared with the OS of the unfavorable genetic profile (Figure 1B). The median OS for the favorable genetic profile was 35.7 months (95% CI: 26.8–44.5 months) versus the 24.3 months of the unfavorable genetic profile (95% CI: 19.7–28.8 months; p = 0.078, log-rank test; Figure 1B). The Cox proportional hazards model, including the same significant parameters described in Table 6, revealed an adjusted HR for the OS of the favorable genetic profile of 0.64 (95% CI: 0.38–1.05; p = 0.081). Of note, the probability of an estimated 1-year survival rate was 91% (95% CI: 84–98) in the favorable genetic profile and 79% (95% CI: 67–91) in the unfavorable genetic profile; the estimated 2-year survival was 72% (95% CI: 60–83) and 51% (95% CI: 36–65), respectively. No significant differences in terms of objective response were observed (73.4% in the favorable genetic profile as compared with 57.4% in the unfavorable genetic profile, p = 0.077).

Also the 56 MBC patients treated with a first-line chemotherapy including paclitaxel without bevacizumab were investigated in order to test the impact of the two genetic profile in both PFS and OS. The results revealed no effect of the favorable genetic profile, as compared with the unfavorable genetic profile, either on the PFS (p = 0.175, log-rank test; Figure 2) or on the OS (p = 0.803, log-rank test; data not shown).

Table 2. Association between clinical and pathological characteristics with progression-free survival and overall survival in 113 patients treated with paclitaxel and bevacizumab.

Characteristics		n (113)	PFS			OS		
			HR	95% CI	p-value	HR	95% CI	p-value
ECOG PS	0	103	1	0.38–1.41	0.354	1	0.17–0.78	0.009
	1	10	0.73			0.36		
Hormonal receptor	Negative	18	1	0.19–0.59	0.0001	1	0.15–0.52	0.0001
	Positive	95	0.34			0.28		
DFI	<12 months	30	1	0.93–2.23	0.106	1	0.55–1.68	0.898
	≥12 months	83	1.44			0.96		
Sites involvement	<3	38	1	0.96–2.2	0.075	1	0.57–1.64	0.890
	≥3	75	1.45			0.96		
Adjuvant CHT	Yes	79	1	0.67–1.55	0.941	1	0.34–1.07	0.081
	No	34	1.02			0.60		
Adjuvant taxanes	Yes	26	1	0.5–1.27	0.333	1	0.19–0.59	0.0001
	No	87	0.79			0.34		
Bevacizumab maintenance	No	30	1	0.33–0.83	0.006	1	0.18–0.49	0.0001
	Yes	83	0.53			0.29		
Visceral disease	Yes	82	1	0.52–1.27	0.363	1	0.57–1.75	0.998
	No	31	0.81			1.00		

CHT: Chemotherapy; DFI: Disease-free interval; ECOG PS: Eastern Cooperative Oncology Group performance status; HR: Hazard ratio; OS: Overall survival; PFS: Progression-free survival.

Discussion

The identification of biomarkers to select patients with the best chance of bevacizumab response is urgently needed to better define the role of this antiangiogenic antibody in the management of MBC patients.

Pander and colleagues have recently suggested the use of MDR as a method to assess the role of genetic polymorphisms in predicting drug response in metastatic colorectal cancer patients [48] treated with capecitabine, oxaliplatin and bevacizumab (CAPOX-B). Particularly, an interaction between *VEGF* +405G>C and *TYMS-TSER* polymorphisms, instead of an individual polymorphism, seemed to predict the CAPOX-B response in terms of PFS, providing a novel pharmacogenetic approach for identifying possible genetic biomarkers of outcome.

In the present analysis, we have applied the MDR methodology for identifying a pharmacogenetic profile associated with a greater PFS as compared with that observed in the whole study population, in an unselected population of patients with MBC treated with fist-line bevacizumab combined to paclitaxel.

We firstly associated each individual SNP with PFS. Although in our analyses the patients with *VEGFR-2* rs2071559 AG genotype, A-variants of *EPAS-1* rs4145836 or *VEGF-A* rs2010963 CC carrier had a greater (but not significant) PFS benefit, caution is needed in the interpretation of these findings. In the last years, several SNPs linked to VEGF-A or VEGFR-2 pathways have been proposed as predictors of bevacizumab response in terms of PFS or OS [10,40]. Of note, none of the SNPs reported have been prospectively evaluated and, significantly, if a SNP appeared to be predictive in one study, it was not generally confirmed as such in subsequent reports. For instance, while a recent analysis conducted in patients with metastatic pancreatic adenocarcinoma revealed a possible predictive role of VEGFR-1 locus for bevacizumab, another did not in MBC patients [14,49]. Therefore, these results have raised doubts about the role of a single polymorphism in predicting bevacizumab response. In addition, because of biological differences between the tumor types, the possibility that some SNPs may be of predictive value in some cancers but not in others, cannot be excluded. Likewise, the individual susceptibility to drug response could depend on the interaction between genes instead of being affected by a single gene polymorphism, as suggested by other authors [48].

For the above reasons, we decided to apply the MDR methodology to investigate the interaction between germline SNPs to identify a possible genetic profile associated with the greater PFS probability in this unselected population of MBC patients treated with bevacizumab combined to first-line paclitaxel (Table 1). The analyses conducted in this unselected

Table 3. Association between clinical and pathological characteristics with progression-free survival in 56 patients treated with paclitaxel alone.

Characteristics		n (56)	PFS		
			HR	95% CI	p-value
ECOG PS	0	40	1		
	1	14	1.09	0.51–2.36	0.82
	2	2	2.37	0.31–18.1	0.41
Hormonal receptor	Negative	5	1	0.32–3.54	0.91
	Positive	51	1.07		
DFI	<12 months	13	1.40	0.66–2.98	0.38
	≥12 months	43	1		
Sites involvement	<3	45	1	0.74–3.62	0.23
	≥3	11	1.63		
Adjuvant CHT	Yes	35	1	0.47–1.81	0.81
	No	21	0.92		
Adjuvant taxanes	Yes	17	1	0.20–0.89	0.02
	No	39	0.43		
Visceral disease	Yes	33	1	0.29–1.16	0.12
	No	23	0.58		

CHT: Chemotherapy; DFI: Disease-free interval; ECOG PS: Eastern Cooperative Oncology Group performance status; HR: Hazard ratio; PFS: Progression-free survival.

Table 4. Association between each polymorphism and progression-free survival in patients treated with paclitaxel and bevacizumab.

Polymorphisms	Genes	Carriers	n	HR	95% CI	p-value
rs699947	*VEGF-A*	AA	17	1		
		AC	57	1.45	0.76–2.74	0.257
		CC	38	1.14	0.59–2.22	0.697
rs833061	*VEGF-A*	CC	17	1		
		CT	56	1.42	0.76–2.68	0.27
		TT	39	1.16	0.6–2.24	0.67
rs3025039	*VEGF-A*	CC	82	1		
		CT	26	1.03	0.64–1.66	0.90
		TT	4	0.47	0.14–1.56	0.22
rs1570360	*VEGF-A*	GG	6	1		
		AG	45	1.59	0.56–4.49	0.38
		AA	61	1.61	0.57–4.51	0.37
rs699946	*VEGF-A*	AA	62	1		
		AG	45	0.89	0.58–1.35	0.57
		GG	5	1.17	0.44–3.16	0.75
rs2010963	*VEGF-A*	GG	43	1		
		CG	56	1.04	0.64–1.62	0.86
		CC	13	0.45	0.22–0.94	0.03
rs2305948	*VEGFR-2*	CC	90	1		
		CT	21	0.87	0.51–1.51	0.63
		TT	1	2.61	0.35–19.4	0.35
rs11133360	*VEGFR-2*	TT	35	1		
		CT	54	0.92	0.58–1.46	0.73
		CC	23	1.07	0.61–1.87	0.82
rs2071559	*VEGFR-2*	AA	25	1		
		AG	55	0.55	0.33–0.93	0.03
		GG	32	0.71	0.41–0.25	0.23
rs1870377	*VEGFR-2*	TT	58	1		
		AT	45	1.23	0.8–1.89	0.34
		AA	9	1.41	0.67–2.96	0.36
rs11549465	*HIF-1α*	CC	8	1		
		CT	100	0.57	0.26–1.22	0.15
		TT	4	0.43	0.12–1.51	0.19
rs2228262	*TSP-1*	AA	98	1		
		AG	12	1.03	0.55–1.96	0.92
		GG	2	1.46	0.35–6.17	0.61
rs4145836	*EPAS-1*	GG	86	1		
		AG	23	0.56	0.33–0.94	0.03

Hormonal receptor status, bevacizumab maintenance and number of sites involvement are the covariates used for the Cox proportional hazards analysis.
A p-value <0.00357 was defined as statistically significant (Bonferroni's correction).
HR: Hazard ratio.