Bevacizumab treatment of patients with breast cancer is associated with induction of AP-1 transcription factor subunits.

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Bevacizumab (Avastin) is an approved treatment option by the European Medicines Agency¹ for more than a quarter billion women in the European Union, and despite having its indication withdrawn by the Food and Drug Administration in 2011 is still utilized in clinical trials in the United States^{2,3}. We mined published microarray data⁴ from the PROMIX trial to understand in an unbiased fashion genes most transcriptionally perturbed by bevacizumab administration and how this interacted with a standard anthracycline and taxane chemotherapeutic regimen, epirubicin and docetaxel. We report here the concomitant induction of AP1 transcription factor subunits JUN, FOS and FOSB in the primary tumors of women with breast cancer treated with bevacizumab, nuclear factors that trigger expression of a host of genes that support the proliferation and growth of cancer⁵⁻¹³.

Keywords: breast cancer, AP-1, FOS, JUN, FOSB, bevacizumab, Avastin, transcription factors in breast cancer, systems biology of breast cancer, targeted therapeutics in breast cancer.

Breast cancer affects women worldwide with relatively high frequency⁸. Bevacizumab (Avastin), a monoclonal antibody targeted against the vascular endothelial growth factor A, VEGF-A, is utilized for the treatment of breast cancer worldwide⁹. Bevacizumab was formerly approved by the Food and Drug Administration in conjunction with paclitaxel for the treatment of metastatic breast cancer which had not previously been treated with chemotherapy for HER2-negative breast cancer; this indication was withdrawn on the basis that its full safety and efficacy had not been demonstrated². For the over quarter of a billion women in the European Union, bevacizumab remains approved by the European Medicines Agency for the treatment of metastatic breast cancer¹. To understand the transcriptional response to bevacizumab in an unbiased fashion at the transcriptome level, we mined published microarray data from PROMIX⁴, a phase II clinical trial measuring clinical responses to epirubicin, docetaxel and bevacizumab in women with breast cancer.

Methods

We used datasets GSE87455⁴, GSE72970¹⁰ and GSE150615¹¹ this global differential gene expression analysis in conjunction with GEO2R. GSE87455 was generated using Illumina HumanHT-12 V4.0 expression beadchip technology with $n=122$ primary breast tumors at baseline, $n=83$ primary tumors after Cycle 2 of treatment, consisting of only chemotherapy (epirubicin and docetaxel) and $n=70$ following addition of bevacizumab to treatment regimen in patients with breast cancer; analysis was performed using platform GPL10558. GSE72970 was generated using Affymetrix Human Genome U133 Plus 2.0 Array with $n=60$ tumors from patients with colorectal cancer receiving FOLFIRI chemotherapy treatment, and $n=22$ tumors from patients with colorectal cancer receiving FOLFIRI and bevacizumab; analysis was performed using platform GPL570. GSE150615 was generated using Genentech glioblastoma custom panel GBM_GNE_C1954 technology with tumors from patients with glioblastoma treated with the following therapeutics: $n=173$ primary tumors from patients treated with placebo (control), $n=116$ primary tumors from patients treated with lomustine, $n=166$ primary tumors from patients treated with bevacizumab, and $n=163$ primary tumors from patients treated with lomustine and bevacizumab; analysis was performed using platform GPL28485. The Benjamini and Hochberg method of p -value adjustment was used for ranking of differential expression but raw p -values were used to assess statistical significance of global differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used. A statistical test was performed to evaluate whether AP-1 transcription factor subunit gene expression was significantly different between primary breast tumors at baseline, following chemotherapy (epirubicin and docetaxel) only and following both chemotherapy and bevacizumab in humans with breast cancer using a one way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. We used PRISM for all statistical analyses of differential gene expression in human breast cancer and colorectal cancer (Version 8.4.0)(455).

Results

We mined published microarray and multiplexed gene expression data^{4,10,11} to understand in an unbiased fashion and at the systems level how transcription globally was most significantly altered in primary tumors from humans with cancer following treatment with the monoclonal antibody bevacizumab.

Bevacizumab treatment results in transcriptional induction of FOS, JUN and FOSB in the primary tumors of women with breast cancer.

We analyzed data from the PROMIX trial⁴ wherein patients were treated for 6 cycles; each of the six cycles included treatment with epirubicin and docetaxel; bevacizumab was added to the treatment

1 regimen only after cycle 2. Tumor tissues were sampled at baseline, following administration of 2 cycles
2 of epirubicin and docetaxel (prior to administration of bevacizumab), and after the addition of
3 bevacizumab, at surgery. We identified the three separate AP-1 transcription factor subunits as among the
4 genes most differentially expressed in the primary tumors of human patients with breast cancer when
5 comparing tumors at baseline, after 2 cycles of epirubicin and docetaxel, and at surgery, once
6 bevacizumab had been administered for 4 cycles: JUN, FOS and FOSB (Table 1, Table 2, and Table 3,
7 respectively). When sorting each of the genes expressed in the primary tumors of humans with breast
8 cancer as compared to baseline, after 2 cycles of epirubicin and docetaxel, and after 4 cycles of
9 bevacizumab, epirubicin and docetaxel, for 6 cycles of total treatment, JUN ranked 9 out of 47323 total
10 transcripts (Table 1). Differential expression of JUN in primary tumors of the breast was statistically
11 significant when comparing tumors at baseline to tumors following treatment with epirubicin and
12 docetaxel or bevacizumab, epirubicin and docetaxel was statistically significant (Table 1: $p=6.61E-30$).
13 When sorting each of the genes expressed in the primary tumors of humans with breast cancer as
14 compared to baseline, after 2 cycles of epirubicin and docetaxel, and after 4 cycles of bevacizumab,
epirubicin and docetaxel, for 6 cycles of total treatment, FOS ranked 6 out of 47323 total transcripts
(Table 1). Differential expression of FOS in primary tumors of the breast was statistically significant
when comparing tumors at baseline to tumors following treatment with epirubicin and docetaxel or
bevacizumab, epirubicin and docetaxel was statistically significant (Table 2; $p=1.58E-32$). When sorting
each of the genes expressed in the primary tumors of humans with breast cancer as compared to baseline,
after 2 cycles of epirubicin and docetaxel, and after 4 cycles of bevacizumab, epirubicin and docetaxel,
for 6 cycles of total treatment, FOSB ranked 18 out of 47323 total transcripts (Table 1). Differential
expression of FOSB in primary tumors of the breast was statistically significant when comparing tumors at
baseline to tumors following treatment with epirubicin and docetaxel or bevacizumab, epirubicin and
docetaxel was also statistically significant (Table 1; $p=4.86E-25$).

Next we evaluated the magnitude and direction of change in AP-1 transcription factor gene
expression in the primary tumors of breast cancer patients after 2 cycles of epirubicin and docetaxel as
well as once bevacizumab had been added to the treatment regimen for 4 cycles. We observed
significantly increased expression of each of these genes, but only after treatment with bevacizumab.
JUN expression significantly increased following addition of bevacizumab to the treatment regimen as
compared to baseline (Figure 1: $p<0.0001$), but JUN expression was not significantly different following
2 cycles of epirubicin and docetaxel as compared to baseline (Figure 1: $p=0.8835$). FOS expression also
significantly increased following addition of bevacizumab to the treatment regimen as compared to
baseline (Figure 2: $p<0.0001$) but again, JUN expression was not significantly different following 2
cycles of epirubicin and docetaxel as compared to baseline (Figure 2: $p=0.7973$). FOSB expression was
also significantly increased following addition of bevacizumab to the treatment regimen as compared to
baseline (Figure 3: $p<0.0001$), but like FOS and JUN, FOSB expression was significantly different
following 2 cycles of epirubicin and docetaxel as compared to baseline (Figure 3: $p=0.9916$).

We calculated a mean fold change of 1.04 ± 0.46 in JUN gene expression when comparing
tumors at baseline to tumors following treatment with 2 cycles of epirubicin and docetaxel, but $2.20 \pm$
 1.02 when comparing tumors at baseline to tumors at following addition of bevacizumab to the treatment
regimen (Table 1). Similarly, we calculated a mean fold change of 1.20 ± 1.65 in FOS gene expression
when comparing tumors at baseline to tumors following treatment with 2 cycles of epirubicin and
docetaxel, but 4.95 ± 4.08 when comparing tumors at baseline to tumors following addition of
bevacizumab to the treatment regimen (Table 2). Likewise, we calculated a mean fold change of $1.04 \pm$
 1.71 in FOSB gene expression when comparing tumors at baseline to tumors following treatment with 2
cycles of epirubicin and docetaxel, but 4.09 ± 4.01 when comparing tumors at baseline to tumors
following addition of bevacizumab to the treatment regiment (Table 3).

JUN is differentially expressed in the tumors of patients with colorectal cancer treated with bevacizumab.

We turned to a second microarray dataset¹⁰ to evaluate transcriptional modulation of AP-1 transcription factors in human cancer, comparing global gene expression between the tumors of patients treated with FOLFIRI chemotherapeutic regimen or the tumors of patients treated with FOLFIRI and bevacizumab. We observed significantly differential expression of JUN in the tumors of patients with colorectal cancer upon treatment with bevacizumab (Table 2). When sorting each of the genes expressed in primary colorectal tumors based on significance of change in expression between FOLFIRI-administered patients treated with bevacizumab or not, JUN ranked 10229 out of 54765 total transcripts (Table 2). Differential expression of JUN in the primary tumors of bevacizumab-treated patients with colorectal cancer was statistically significant (Table 3; $p=1.91E-02$).

FOS is differentially expressed in the tumors of patients with glioblastoma following treatment with bevacizumab.

We studied a third dataset, this multiplexed gene expression data generated using tissues from patients with glioblastoma treated with placebo, lomustine, bevacizumab, or both lomustine and bevacizumab, as well tumor tissues from placebo-treated patients, to further evaluate differential expression of AP-1 components in human cancer¹¹. We found that in glioblastoma, FOS was also a differentially expressed gene when comparing patient tumors based on treatment (Table 4). When sorting each of the genes expressed in glioblastoma tumors based on significance of change in expression between tumors of placebo-treated patients and the tumors of patients treated with either lomustine, bevacizumab, or lomustine and bevacizumab, FOS ranked 383 out of 786 total transcripts (Table 3), equating to 51.3% differential expression. Differential expression of FOS in the tumors of glioblastoma patients treated with either lomustine, bevacizumab, or lomustine and bevacizumab was statistically significant (Table 3; $p=1.04E-23$).

We then asked whether differential expression of FOS was attributable to treatment with bevacizumab or with lomustine, by comparing tumor transcriptomes between those treated with placebo and those treated with bevacizumab, as well as those treated with placebo and those treated with lomustine. While FOS was the 153rd differentially expressed transcript out of 786 when comparing tumors of placebo-treated patients to tumors of bevacizumab-treated patients, with differential expression equating to 80.5%, FOS was the 347th differentially expressed transcript out of 786 when comparing tumors of placebo-treated patients to tumors from lomustine-treated patients, equating to only 55.9% differential expression. Thus, we conclude that differential expression of FOS in the tumors of patients with glioblastoma was the result of treatment with bevacizumab.

Thus, through blind, systems-level transcriptome analysis of primary tumors from patients with breast cancer patient, we found that JUN, FOS and FOSB - three separate AP-1 transcription factor subunits - were among the genes most differentially expressed in the primary tumors of patients treated with a regimen including epirubicin, docetaxel and bevacizumab. However, in each case, transcriptional induction of AP-1 transcription factor subunits did not occur until bevacizumab administration was initiated. JUN and FOS were also among the genes most differentially expressed upon treatment with bevacizumab in the primary tumors of patients with colorectal cancer and glioblastoma. Together, we conclude that at least in human breast cancer, bevacizumab results in transcriptional induction of multiple AP-1 transcription factor subunits.

Discussion

FOS and JUN heterodimerize to form the AP-1 transcription factor and trans activate the expression of a network of AP-1 responsive genes through site-specific DNA binding^{5,6}. AP-1 was originally described as an oncoprotein⁶. AP-1 can support expression of genes necessary for cell survival and for cellular proliferation including Cyclin D1 and E2F⁷⁻¹⁰. Three canonical pathways that are activated by AP-1 are the MAP kinase pathways ERK, p38 MAPK and c-Jun N-terminal kinase JNK⁷.

The relevance of the AP-1 gene expression program and the ability of AP-1 subunits JUN and FOS to support cellular proliferation and survival in the context of cancer is evidenced by studies demonstrating that expression of a dominant negative mutant of c-JUN, TAM67, could block proliferation of MCF-7 breast cancer cells induced by serum¹¹. Dominant negative c-JUN could also block proliferation of MCF-7 cells in response to IGF-1, EGF and bFGF, and partially block proliferation in provoked by estrogen¹¹. A similar response was observed when MCF-7 cells were injected into nude mice to assess tumor growth *in vivo* in response to AP-1 inhibition by dominant negative c-JUN¹¹. As in non-cancerous cellular contexts, AP-1 could support proliferation of breast cancer cells and progression through the cell cycle by activating expression of Cyclin D1 and E2F1 and E2F2 transcription factors¹². At the functional level, AP-1 transcriptional activity is associated with resistance to tamoxifen and early relapse of metastasis¹³.

We found here that AP-1 transcription factor subunits JUN, FOS and JUNB are among the genes whose expression changes most significantly in primary tumors of the breast upon treatment with bevacizumab. JUN, FOS and JUNB were all expressed at significantly higher levels in primary tumors of the breast following treatment with bevacizumab but not following treatment with epirubicin and docetaxel chemotherapy. In summary, we provided evidence here that bevacizumab induces expression of multiple components of a central transducer of growth factor and mitogen-activated proliferation signals shown to support *in vitro* and *in vivo* growth of tumor cells in breast cancer.

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Rank	ID	p-value	F	FC	Gene	Gene name
9	ILMN_1806023	6.61E-30	8.68E+01	B+E: 1.04 ± 0.46 B+E+D: 2.20 ± 1.02	JUN	Jun proto-oncogene, AP-1 transcription factor subunit

Table 1: Differential expression of JUN in the primary breast tumors of women treated with bevacizumab.

The rank of differential expression, probe ID, *p*-value with respect to global differential expression, F-statistic, fold change in JUN transcription factor gene expression between primary tumors at baseline and primary tumors following administration of chemotherapy only (“E+D”: epirubicin and docetaxel), fold change in JUN gene expression between primary tumors at baseline and primary tumors following addition of bevacizumab to epirubicin and docetaxel (“B+E+D”), gene and gene name are listed in this chart.

Rank	ID	p-value	F	FC	Gene	Gene name
6	ILMN_1669523	1.58E-32	9.68E+01	B+E: 1.20 ± 1.65 B+E+D: 4.95 ± 4.08	FOS	Fos proto-oncogene, AP-1 transcription factor subunit

Table 2: Differential expression of FOS in the primary breast tumors of women treated with bevacizumab.

The rank of differential expression, probe ID, *p*-value with respect to global differential expression, F-statistic, fold change in FOS transcription factor gene expression between primary tumors at baseline and primary tumors following administration of chemotherapy only (“E+D”: epirubicin and docetaxel), fold change in FOS gene expression between primary tumors at baseline and primary tumors following addition of bevacizumab to epirubicin and docetaxel (“B+E+D”), gene and gene name are listed in this chart.

Rank	ID	p-value	F	FC	Gene	Gene name
18	ILMN_1751607	4.86E-25	6.92E+01	<u>B+E</u> : 1.04 ± 1.71 <u>B+E+D</u> : 4.09 ± 4.01	FOSB	FosB proto-oncogene, AP-1 transcription factor subunit

Table 3: Differential expression of FOSB in the primary breast tumors of women treated with bevacizumab.

The rank of differential expression, probe ID, *p*-value with respect to global differential expression, F-statistic, fold change in FOSB transcription factor gene expression between primary tumors at baseline and primary tumors following administration of chemotherapy only (“E+D”: epirubicin and docetaxel), fold change in FOSB gene expression between primary tumors at baseline and primary tumors following addition of bevacizumab to epirubicin and docetaxel (“B+E+D”), gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
10229	201464_x_at	1.91E-02	2.3897649	-3.38719	JUN	Jun proto-oncogene, AP-1 transcription factor subunit

Table 4: Differential expression of JUN in colorectal tumors of patients treated with bevacizumab.

The rank of differential expression, probe ID, *p*-value with respect to global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the two groups compared, gene and gene name are listed in this chart.

Rank	transcript ID	p-value	F	Gene	% DE
383	NM_005252.2	1.04E-23	40.1485	FOS	51.3%

Table 5: FOS is differentially expressed in the tumors of patients with glioblastoma when comparing primary tumor gene expression based on treatment with either lomustine, bevacizumab, or both lomustine and bevacizumab.

The rank of differential expression, transcript ID, p-value with respect to global differential expression, F statistic, and gene symbol are listed in this chart.

Rank	transcript ID	p-value	t	B	Gene	% DE
153	NM_005252.2	0.31026	1.0162	-4.66	FOS	80.5%

Table 6: FOS is among the top 20% of differentially expressed genes in the tumors of patients with glioblastoma when compared based on treatment with placebo or bevacizumab.

The rank of differential expression, transcript ID, p-value with respect to global differential expression, F statistic, and gene symbol are listed in this chart.

Rank	transcript ID	p-value	t	B	Gene	% DE
347	NM_005252.2	5.65E-14	-7.9048	20.374	FOS	55.9%

Table 7: When comparing tumor gene expression in glioblastoma based on treatment with placebo or lomustine, FOS is only marginally differentially expressed, at the 50-60th percentile.

The rank of differential expression, transcript ID, p-value with respect to global differential expression, F statistic, and gene symbol are listed in this chart.

Figure 1: JUN is transcriptionally induced in primary tumors of the breast following addition of bevacizumab to epirubicin and docetaxel treatment but not following treatment with epirubicin and docetaxel only in patients with breast cancer.

Expression of JUN at the messenger RNA level from the tumors of patients enrolled in the PROMIX trial is graphically depicted here in primary tumors at baseline (left), in primary tumors following administration of 2 cycles of chemotherapy only (epirubicin and docetaxel) (“Chemo”, middle), and in primary tumors at surgery, following addition of bevacizumab to treatment regimen (“Chemo + Bev”, right), with mean JUN mRNA level marked and the result of a statistical test evaluating significance of difference in JUN expression between tumors at baseline and tumors following chemotherapy or tumors following addition of bevacizumab to chemotherapy, a *p*-value, listed above. ****: $p < 0.0001$. ns: not significant ($p = 0.8835$).

Figure 2: FOS is transcriptionally induced in primary tumors of the breast tumors following addition of bevacizumab to epirubicin and docetaxel treatment but not following treatment with epirubicin and docetaxel only in patients with breast cancer.

Expression of FOS at the messenger RNA level from the tumors of patients enrolled in the PROMIX trial is graphically depicted here in primary tumors at baseline (left), in primary tumors following administration of 2 cycles of chemotherapy only (epirubicin and docetaxel) (“Chemo”, middle), and in primary tumors at surgery, following addition of bevacizumab to treatment regimen (“Chemo + Bev”, right), with mean FOS mRNA level marked and the result of a statistical test evaluating significance of difference in FOS expression between tumors at baseline and tumors following chemotherapy or tumors following addition of bevacizumab to chemotherapy, a p -value, listed above. ****: $p < 0.0001$. ns: not significant ($p = 0.7973$).

Figure 3: FOSB is transcriptionally induced in primary tumors of the breast following addition of bevacizumab to epirubicin and docetaxel treatment but not following treatment with epirubicin and docetaxel only in patients with breast cancer.

Expression of FOSB at the messenger RNA level from the tumors of patients enrolled in the PROMIX trial is graphically depicted here in primary tumors at baseline (left), in primary tumors following administration of 2 cycles of chemotherapy only (epirubicin and docetaxel) (“Chemo”, middle), and in primary tumors at surgery, following addition of bevacizumab to treatment regimen (“Chemo + Bev”, right), with mean FOSB mRNA level marked and the result of a statistical test evaluating significance of difference in FOSB expression between tumors at baseline and tumors following chemotherapy or tumors following addition of bevacizumab to chemotherapy, a *p*-value, listed above. ****: $p < 0.0001$. ns: not significant ($p = 0.9916$).