Bevacizumab treatment of patients with breast cancer results in induction of KLF2, KLF4, KLF6 and KLF9.

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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Medicines Agency¹ for more than a quarter billion women in the European Union, and despite having its indication withdrawn by the Food and Drug Administration in 2011 is still utilized in clinical trials in the United States^{2,3}.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 striking induction of four separate KLF transcription factors in the primary tumors of women treated with bevacizumab: KLF2, KLF4, KLF6 and KLF9, two of which have established roles in the generation or maintenance of ground state pluripotency in embryonic stem cells^{5,6}.

Keywords: breast cancer, KLF2, KLF4, KLF6, KLF9, 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 frequency⁷. Bevacizumab (Avastin), a monoclonal antibody targeted against the vascular endothelial growth factor A, VEGF-A, is utilized for the treatment of breast cancer worldwide⁸. 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cancer which had not previously been treated with chemotherapy for HER2-negative breast cancer; this indication was withdrawn on the basis that its full safety and efficacy had not been demonstrated².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 and GSE18195⁹ for 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), $n=70$ following addition of bevacizumab to treatment regimen in patients with breast cancer, and $n=166$ primary tumors from patients treated with bevacizumab; analysis was performed using platform GPL570; analysis was performed using platform GPL10558. GSE18195 was generated using Affymetrix Human Genome U133 Plus 2.0 Array with $n=2$ primary tumors from mice untreated (control) and $n=2$ primary tumors from patients treated with lomustine. 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 KLF transcription factor 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. A statistical test was used to evaluate whether KLF8 gene expression was significantly different in the endometrial tumors of mice treated or not with bevacizumab using an unpaired t-test. We used PRISM for all statistical analyses of differential gene expression in human breast cancer and murine endometrial cancer (Version 8.4.0)(455).

Results

We mined published microarray data^{4,9} to understand in an unbiased fashion and at the systems level how transcription globally was most significantly altered in primary tumors from humans and from mice following treatment with the monoclonal antibody bevacizumab.

Bevacizumab treatment results in transcriptional induction of multiple KLF transcription factors 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 regimen only after cycle 2. Tumor tissues were sampled at baseline, following administration of 2 cycles of epirubicin and docetaxel (prior to administration of bevacizumab), and after the addition of

1 bevacizumab, at surgery. We identified the Kruppel-like transcription factors KLF2, KLF4, KLF6 and
2 KLF9 as among the genes most differentially expressed in the primary tumors of human patients with
3 breast cancer when comparing tumors at baseline, after 2 cycles of epirubicin and docetaxel, and at
4 surgery, once bevacizumab had been administered for 4 cycles (Table 1). When sorting each of the genes
5 expressed in the primary tumors of humans with breast cancer as compared to baseline, after 2 cycles of
6 epirubicin and docetaxel, and after 4 cycles of bevacizumab, epirubicin and docetaxel, for 6 cycles of
7 total treatment, KLF2 ranked 158 out of 47323 total transcripts, KLF4 ranked 132 out of 47323 total
8 transcripts, KLF6 ranked 20 out of 47323 total transcripts, and KLF9 ranked 17 out of 47323 total
9 transcripts. Differential expression of KLF2 (Table 1; $p=8.28E-16$), KLF4 (Table 1; $p=1.33E-16$), KLF6
10 (Table 1; $p=5.22E-25$) and KLF9 (Table 1; $p=4.34E-25$) in the primary tumors of women with breast
11 cancer compared at baseline, after 2 cycles of epirubicin and docetaxel, and after 4 cycles of
12 bevacizumab, epirubicin and docetaxel was statistically significant.

13
14 Next we evaluated the magnitude and direction of change in KLF transcription factor gene
15 expression in the primary tumors of patients treated with bevacizumab, after 2 cycles of epirubicin and
16 docetaxel as well as once bevacizumab had been added to the treatment regimen for 4 cycles.
17 Remarkably, we found that each of these transcription factors significantly increased in expression, but
18 only after treatment with bevacizumab (Figure 1, Figure 2, Figure 3 and Figure 4; $p<0.0001$ for each KLF
19 factor following addition of bevacizumab to chemotherapy as compared to baseline). Neither KLF2,
20 KLF4, KLF6 or KLF9 increased in gene expression following treatment with epirubicin and docetaxel; in
21 fact, KLF2 and KLF9 decreased in gene expression following treatment with epirubicin and docetaxel
22 (Figure 1 and Figure 4). We calculated a mean fold change of 0.84 ± 0.27 in KLF2 and 0.77 ± 0.31 in
23 KLF9 gene expression in primary tumors following 2 cycles of epirubicin and docetaxel chemotherapy,
24 but a mean fold change of 1.49 ± 0.56 in KLF2 and 2.04 ± 1.02 in KLF9 gene expression following
25 addition of bevacizumab to the treatment regimen (Table 1). We calculated a mean fold change of $1.01 \pm$
26 0.34 in KLF6 and 0.98 ± 0.60 in KLF4 gene expression in primary tumors following 2 cycles of
27 epirubicin and docetaxel chemotherapy, but a mean fold change of 1.01 ± 0.34 in KLF6 and 0.98 ± 0.60
28 in KLF4 gene expression following addition of bevacizumab to the treatment regimen (Table 1).

17 **Bevacizumab treatment results in transcriptional induction of KLF8 in the endometrial tumors of** 18 **mice.**

19 We turned to a second microarray dataset⁹ to understand more thoroughly tissue-specific
20 transcriptional modulation of bevacizumab in cancer, comparing global gene expression between the
21 untreated tumors of murine endometrial tumors and the endometrial tumors of mice treated with
22 bevacizumab. We found that a KLF transcription factor, KLF8 was among the genes most differentially
23 expressed when comparing bevacizumab-treated murine endometrial tumors to untreated murine
24 endometrial tumors (Table 2). When sorting each of the genes expressed in murine endometrial tumors
25 based on significance of change in expression between untreated and bevacizumab-treated mice, KLF8
26 ranked 54 out of 54675 total transcripts (Table 2). Differential expression of KLF8 in bevacizumab-
27 treated murine endometrial tumors was statistically significant (Table 2; $p=0.00076$).

28 We also assessed magnitude and direction of KLF8 transcriptional modulation by bevacizumab in
tumors of the endometrium in mice. Just as we observed marked induction of KLF2, KLF4, KLF6 and
KLF9 in the primary breast tumors of women treated with bevacizumab, we witnessed transcriptional
induction of KLF8 in the primary endometrial tumors of mice treated with bevacizumab (Figure 5). We
calculated a mean fold change of 14.73 ± 1.98 in KLF8 gene expression in the endometrial tumors of
mice treated with bevacizumab as compared to primary tumors of untreated mice. Transcriptional
induction of KLF8 in the primary tumors of mice treated with bevacizumab was statistically significant
(Figure 5; $p=0.0105$).

Discussion

KLF4 in conjunction with Oct4 can induce pluripotency⁵, and KLF2 is important for maintenance of ground state pluripotency⁶. KLF4 has also been shown to be important for the maintenance of breast cancer stem cells¹⁰ and nuclear localization of KLF4, consistent with actual transcriptional function of KLF4, is associated with aggressive disease behavior in early-stage breast cancer¹¹. KLF9 can function in the oxidative stress response¹², which can be mutagenic. A variant transcript of KLF6 is associated with metastasis and inferior survival in breast cancer¹³, and in renal carcinoma KLF6 drives tumor growth¹⁴.

We provided evidence here that multiple KLF transcription factors are concomitantly up-regulated in the tumors of patients with breast cancer following treatment with the monoclonal antibody bevacizumab. Up-regulation of KLF transcription factors by bevacizumab, two of which are important in the induction and maintenance of pluripotency, should serve as an alarm, to elicit further investigation into the mechanisms by which bevacizumab functions at the molecular level, a drug which has been deemed to lack safety and efficacy in the country in which it was produced and first approved but is now being utilized in other localities.

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Rank	ID	p-value	F	FC	Gene	Gene name
17	ILMN_1778523	4.34E-25	6.93E+01	E+D: 0.77 ± 0.31 B+E+D: 2.04 ± 1.02	KLF9	Kruppel like factor 9
20	ILMN_1735014	5.22E-25	6.91E+01	E+D: 1.01 ± 0.34 B+E+D: 1.80 ± 0.66	KLF6	Kruppel like factor 6
132	ILMN_2137789	1.33E-16	4.19E+01	E+D: 0.98 ± 0.60 B+E+D: 1.89 ± 1.04	KLF4	Kruppel like factor 4
158	ILMN_1735930	8.28E-16	3.95E+01	E+D: 0.84 ± 0.27 B+E+D: 1.49 ± 0.56	KLF2	Kruppel like factor 2

Table 1: Differential expression of multiple KLF transcription factors 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 KLF 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 KLF transcription factor gene expression between primary tumors at baseline and primary tumors following addition of bevacizumab to epirubicin and docetaxel (“B+E+D”: bevacizumab, epirubicin and docetaxel) gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
54	219930_at	0.00076	7.5574269	-0.56141	14.73 ± 1.98	KLF8	Kruppel like factor 8

Table 2: Differential expression of KLF8 in endometrial tumors of mice 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, fold change in KLF8 gene expression between tumors at baseline and tumors following administration of bevacizumab, gene and gene name are listed in this chart.

Figure 1: KLF2 is transcriptionally induced in the breast tumors of women following addition of bevacizumab to epirubicin and docetaxel treatment but not following treatment with epirubicin and docetaxel only.

Expression of KLF2 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 KLF2 mRNA level marked and the result of a statistical test evaluating significance of difference in KLF2 expression between primary tumors of the breast based on the presence or absence of bevacizumab in the treatment regimen, a p-value, listed above. ****: $p < 0.0001$. *: $p = 0.0112$

Figure 2: KLF4 is transcriptionally induced in the breast tumors of women following addition of bevacizumab to epirubicin and docetaxel treatment but not following treatment with epirubicin and docetaxel only.

Expression of KLF4 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 KLF4 mRNA level marked and the result of a statistical test evaluating significance of difference in KLF4 expression between primary tumors of the breast based on the presence or absence of bevacizumab in the treatment regimen, a p-value, listed above. *****: $p < 0.0001$. ns: not significant.

Figure 3: KLF6 is transcriptionally induced in the breast tumors of women following addition of bevacizumab to epirubicin and docetaxel treatment but not following treatment with epirubicin and docetaxel only.

Expression of KLF6 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 KLF6 mRNA level marked and the result of a statistical test evaluating significance of difference in KLF6 expression between primary tumors of the breast based on the presence or absence of bevacizumab in the treatment regimen, a p-value, listed above. *****: $p < 0.0001$. ns: not significant.

Figure 4: KLF9 is transcriptionally induced in the breast tumors of women following addition of bevacizumab to epirubicin and docetaxel treatment but not following treatment with epirubicin and docetaxel only.

Expression of KLF9 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 KLF9 mRNA level marked and the result of a statistical test evaluating significance of difference in KLF9 expression between primary tumors of the breast based on the presence or absence of bevacizumab in the treatment regimen, a p-value, listed above. *****: $p < 0.0001$. *: 0.0194.

Figure 5: Bevacizumab treatment results in transcriptional induction of KLF8 in the endometrial tumors of mice.

Expression of KLF8 at the messenger RNA level in murine endometrial tumors not treated (left), and in murine endometrial tumors from mice treated with bevacizumab (right), with mean KLF8 mRNA level marked and the result of a statistical test evaluating significance of difference in KLF8 expression between the endometrial tumors of untreated and bevacizumab-treated mice, a p-value, listed above.