Author's response to reviews

Title: Dermcidin exerts its oncogenic effects in breast cancer via modulating ERBB signaling

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Author's response to reviews: see over
Author's response to reviews

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Version: 2
Date: 6 November 2014

Author's response to reviews: see over
Dear Dr Vadlamudi

We would like to thank the reviewers and Editors of BMC Cancer for considering the manuscript we submitted. We are re-submitting a revised version of the manuscript entitled “Dermcidin exerts its oncogenic effects in breast cancer via modulating ERBB signaling”, taking into consideration all comments and suggestions made by the reviewers. We have also extensively revised the text, deleted and/or rewritten with minor modifications few sentences, as well as, we have performed and added additional figure (Figure 7), additional file 6: S3 ppt and reference papers to further support of our conclusions. The text alterations are typed on blue. We fell that we have satisfactorily dealt with the issues raised by reviewers and would appreciate your review of this revised manuscript. Thank you very much for your consideration.

In reply to the reviewers’ general and specific comments and questions, we have answered point-to-point to each one as follows:

**Reviewer: Roxana Schillaci**

We agree with reviewer’s observations and comments. Considering the various critics and suggestions, we have re-written parts of text to include the comments and observations. We also included a new figure and additional file to further support the final conclusion of this manuscript.

**Major Revision**

We agree with many points raised by this reviewer. There are few studies published in literature reporting on possible molecular mechanism by which DCD native protein and DCD-generated peptides exert its function as growth and survival factor and antibiotic peptide (see review article: Schittek, J Innate Immun 4:349-360). Our previous studies showed that presence of low and high affinity cell surface receptors to DCD in breast cancer cell lines (Porter et al., 2003). We believe that the presence of these membrane receptors would direct
or indirectly promote the integration of network signaling pathway leading to EGFR phosphorylation and activation of p38 MAPK and Akt, as showed in the figure 5 and 6 of the Manuscript. Nonetheless, a recent study has described the crystal and atomic structure of DCD peptide and detailed mechanism by which individual peptides undergo oligomerization and assembly as channel structure channel structure with ion conductivity properties across biomimetic membrane (Song et al., PNAS 10(12): 4586, 2013). We have not explored these mechanisms in our study, which is focused on the biological consequences of DCD up or down regulation in human breast cancer cell lines. By using a genomic approach, we have identified 208 up and 27 down-regulated genes modulated by DCD, including the HER/ErbB family genes. The molecular mechanism and functional activities of these genes are well known (Citri A, Yarden Y, Nat Rev Mol Cell Biol 7:505-16, 2006), thus we have proposed the ERBB signaling modulation as the mechanism to explain DCD effects on cell proliferation, resistance to cell death, and tumor growth in immunodeficient mice.

Major Points:

1. We have done the additional experiments to investigate the effects of polyclonal goat antibodies anti-DCD and Herceptin (trastuzumab), a humanized monoclonal antibody against HER2, on MDA-MB-361 cell colony formation in cell culture and tumor growth as xenograft in immunodeficient mice, as suggested by this reviewer. The results of these studies are presented on figure 7 of the revised version of the manuscript. The data showed that the treatment of mice with the anti-DCD antibodies or the combination of anti-DCD polyclonal antibodies and trastuzumab caused a significant reduction in the number of colonies in cell culture as well as the MDA-MB-361 tumor growth in NUDE mice. These results suggest that DCD molecules autocrinely produced by MDA-MB-361 cells and act as growth factor and cooperate with HER/ErbBs ligands to stimulate the growth and proliferation of these cells in vitro and in vivo.

2. We have done the additional experiments to investigate whether the overexpression of DCD gene in SK-BR-3 cells increase the proliferation of this HER2-amplified cell line when implanted in the mammary fat pads of female immunodeficient mice. The data presented as additional file 6: figure S3 showed that overexpression of DCD increase the tumor growth in NUDE mice as observed in experiments using MCF-7 breast cancer cell line.

3. In this study we have examined the levels and pattern of DCD expression by IHC de-identified cores (TMA) from more than 600 breast carcinoma patients. The clinical and pathological features of the cohort of patients have been published previously (Logullo et al., 2011, Histopathology, 58:617-625, 2011). The most majority of tumor cells were negative to DCD expression. Statistically significant associations (p<0.05) were found between tumor samples having DCD reactivity >50% of tumor cells and the subgroups with either high histological or with HER2 score 3 (Table 1). Next, we explored and confirmed this association using the microarray data sets of 55 human breast cancer cell lines obtained from Cancer Cell Line Encyclopedia. We found statistically significant association (p<0.05) between DCD expression (RMA ≥4) with HER2 (RMA ≥8) and also with HER3 (RMA ≥9) expression (Table 1.B). The association of DCD and HER2/ErbB2 at transcriptional regulation was not confirmed in MDA-MB-361 cell line because it is HER2-amplified cell line and contains multiple copies of her2 gene (Neve et al., 2006). The constitutive autocrine expression of DCD significantly increased the mRNA levels of EGFR and HER2/ErbB2 in MCF-7 cell line, which is HER2 non-amplified cell line (Figure 6E, F).
4. Our previous studies using serial analysis of gene expression (SAGE) and immunohistochemistry in specimens of over 675 patients with breast tumors in different histological stages confirmed that DCD is overexpressed in less than 10%, mainly in invasive breast carcinoma samples (Porter et al., 2003a, PNAS, 100:10931-6, 2003). In this study we have examined the levels and pattern of DCD expression by IHC in more than 600 tissue samples arrayed in TMA. A group of 26 samples (approximately 5%) with consistently DCD immunoreactivity in <50% or >50% of tumor cells was classified into subgroups according to their clinical and pathological features. Statistically significant associations (p<0.05) were found between tumor samples with DCD reactivity >50% and the subgroups with either high histological grade or with HER2 expression at score 3 level (Table 1A). No relationship with overall survival was found. These results are in line with the findings obtained in the studies with breast cancer cell line.

5. In the Western Blot showed on figures 5 and 6, the expression of the α-tubulin was used as loading control of the SDS-PAGE since an equivalent amount of protein (fifty µg of protein from cellular lysate) of untreated and treated samples were subjected to pEGFR, EGFR, p38MAPK and pAkt immunoblot analyses. The lanes of blot show the total protein expression for EGFR, and only immune reactive bands correspondent to pEGFR, p38MAPK and Akt phosphorylated forms, which were detected by specific antibodies using chemiluminescence. These antibodies do not recognize the un-phosphorylated forms of proteins as described in Material and Methods.

6. We have not evaluated HER2/ErbB-2 protein phosphorylation in MDA-MB-361 pLKO clone (pLKO group) or MDA-MB-361 DCD shRNA clone (IBC-I group) in this study. The variation of protein levels for EGFR, HER2/ErbB-2 and HER4 were investigated by immunohistochemical in tissue tumors samples from mice bearing xenografts of MDA-MB-361 pLKO (pLKO group) and MDA-MB-361 DCD shRNA clone (IBC-I group) cells. These results are provided as Additional file 4: Fig. 1. If the reviewer finds this additional file is relevant for the critical analysis of our results, the additional file can be transferred to figures of the revised version of manuscript.

7. Our previous study showed biochemical evidence that DCD may induce its cell proliferation and survival activities by binding to the high and low affinity membrane receptors expressed in breast cancer cell lines (Porter et al., 2003, PNAS 100(19): 1091, 2003). A recent study has shown that individual DCD-1L antibiotic peptides can undergo oligomerization as trimer to form hexameric structure that may function as conductive channel across lipid bilayers (Song et al., PNAS 10(12): 4586, 2013). In discussion section of the revised version of manuscript, we have presented this finding and suggested as possible mechanism for DCD action in breast cancer cells.

Minor points

1. The sentence “PCR, microarray..” in Material and Methods has been reworded as suggested.

2. We have re-written the section “Xenograft assays in immunodeficient mice” in Material and Methods to describe the experimental assay for testing the effects of goat polyclonal antibodies against DCD and trastuzumab (Herceptin) against HER2 receptor in MDA-MB-361 tumor growth in immunodeficient mice. The results of this study are presented in figure 7 in the revised version of the manuscript.
Reviewer: Samaya Krishnan

We agree with reviewer’s observations and comments. Considering the various critics and suggestions, we have re-written parts of text to include the comments and observations. We performed additional experiments and included new data to support the final conclusions of this manuscript, as suggested by this reviewer.

Major compulsory revisions

1- The article published in Genet Mol Res 2008, Sept 2007, 7(3): 925-32 is now cited in text of revised version of the manuscript. The DNA microarray analysis done in previous study was re-evaluated using different bioinformatics tools described in Material and Methods. The microarray dataset named “the MDA-MB-361 human breast cancer cell line expressing DCD-targeting shRNA in pLKO vector (clones 1, 2 and 3) and pLKO control vector” has been deposited at GEO, series GSE57578. In the present manuscript we show the results of experimental validation of a group of genes using real time PCR, and the results of biochemical and immunohistochemistry analyses in tissue tumors and cellular and animal models to provide further evidence that DCD is a candidate onco gene in a subset of breast tumors. The results extended findings of previous studies which were done in a smaller cohort of patients and small number of breast cancer cell lines (Porter et al., 2003, PNAS 100(19): 1091, 2003).

2- This manuscript describes at first time the co-expression of DCD and DCD-SV in normal skin tissue and breast cancer cell lines using validated and novel specific antibodies to different portion of the proteins. The DCD spliced variant is identical in its nucleotide sequence to larger (DCD-SV-2) form of DCD identified in human placental tissue as described by Motoyama and colleagues (Motoyama et al., Bioch Biophyc Res Commun 357: 828, 2007). We also found that DCD-SV transcript and proteins are expressed in placenta (data not shown). We designed double-stranded oligonucleotide against different exon sequences of DCD and cloned them in three specific shRNA lentivirus vectors. We analyzed the expression of DCD and DCD-SV transcripts by PCR in MDA-MB-361 cell clones transfected with IBC-I, II and III vectors and found that ICB-I vector containing a sense 5'-CCG GTC CTA GAT CCC AAG ATC TCC AAC TCG AGT TGG AGA TCT TGG GAT CTA GGT TTT TTG-3', anti-sense 5'-AAT TCA AAA ACC TAG ATC CCA AGA TCT CCA ACT CGA GTT GGA GAT CTT GGG ATC TAG GA-3' oligonucleotide sequence was able to block the expression of both DCD and DCD-SV transcripts. So, this vector was used in the assays intended to known-down the DCD expression in cell lines. Thus, we cannot conclude whether DCD-SV has the same effects on the expression of HER/ErbB family of receptors and ligands from the analysis of data presented in this study.

3. In this study we have not investigated the pharmacological effects of EGFR tyrosine kinase inhibitors, and nor the effects of small inhibitors of kinases PI3K/AKT and MAPK of downstream signaling pathways. The use of these molecules would clearly very important to elucidate the biological processes behind DCD mechanism of action. Our results using PCR analysis are very clear to show that knockdown or addition of recombinant DCD protein promote down or up-regulation on the expression of HER2/ErbB receptors and their ligands, in particular, EGFR. It is possible to conclude at the visual examination that the bands in lane 3 and 4 were slightly reduced as compared to band 1 and 2 in Figure 6G. These differences are more emphasized in the slides of additional file 4:Fig. S1. The number and intensity immunohistochemical staining of the EGFR positive cells of tumor tissues from xenografts of MDA-MB-361 pLKO clone (pLKO group) were reduced in MDA-MB-361 DCD shRNA clone (IBC-I group). If the reviewer finds this additional file is relevant for the critical analysis of results of this study, it can be transferred to figures of the revised version of the manuscript.
Finally, it is important to mention a recent study published by Wilhelm and colleagues (Nature, Volume 509, page 582-587) which confirmed the biological role of DCD to cellular resistance to EGFR/ErbB inhibitors. Figure 5B of Wilhelm’s paper displays a list of genes (biomarkers) that confer resistance against EGFR kinase inhibitors, in particular, Erlotinib and lapatinib. The analysis at protein and mRNA levels confirmed that DCD is one top factor conferring cellular resistance to many tumor cell lines. These results provide a new avenue to investigate the survival activity of DCD at molecular level.

4. We have performed additional experiments and included new data (Figure 7) in the revised version of the manuscript to provide further support for the hypothesis that DCD modulates EGFR/HER family of proteins. We have tested the efficacy of the trastuzumab, a humanized polyclonal antibody to HER2, and goat polyclonal antibodies to DCD for the treatment of MDA-MB-361 tumor xenografts in NUDE mice. As shown in figure 7, the results demonstrated that the treatment of mice with the anti-DCD antibodies and combination of trastuzumab caused a significant reduction of the number of colonies of cells in culture as well as tumor growth as xenograft in NUDE mice. These results confirm our hypothesis that DCD autocrinally produced by MDA-MB-361 cells may be acting in concert with the ligands of HER/ErbB receptor family to stimulate the growth and proliferation of breast cancer cells.

Minor essential revisions

5. We have made the corrections on numbers and legends on Figure 3E and 5A and other figures and revised the text of new version of the manuscript.

6. The conclusions regarding the expression of EGF and NRG1 presented in Figure 5A are stated correctly in the text of revised version of the manuscript.

7. In the Western Blot showed on figures 5 and 6, the expression of the α-tubulin was used as loading control of the SDS-PAGE since an equivalent amount of protein (fifty µg of protein of cellular lysate) of untreated and treated samples were subjected to pEGFR, EGFR, p38MAPK and pAkt immunoblot analyses. The lanes of blot show the total protein expression for EGFR, and only immune reactive bands correspondent to EGFR, p38MAPK and Akt phosphorylated forms, which were detected by specific antibodies using chemiluminescence. These antibodies do not recognize the un-phosphorylated forms of proteins as described in Material and Methods.

8. We have revised and rewritten and included additional text in the Material and Methods section.

Reviewer: Paraic Kenny

We agree with this reviewer’s observations and comments. Considering the various critics and suggestions, we have re-written parts of text to include the comments and observations.

General Comments

In the revised version of manuscript we present additional data to further support the major conclusion of this study concerning the cooperation of DCD with EGFR/ErbB ligands in the stimulation of breast cancer growth and survival in vitro and in vivo. The results of these studies are presented in figure 7 and additional file 6: S3 ppt. We investigated the pharmacological effects of polyclonal goat antibodies anti-DCD and Herceptin (trastuzumab), a humanized monoclonal antibody against HER2, in MDA-MB-361 cancer cells. The data showed
that the addition of these antibodies to cell culture reduced the number of colonies and the weekly injection anti-DCD antibodies or the combination of anti-DCD and Herceptin caused a significant reduction of the tumor growth in NUDE mice. The results confirms that DCD autocrinally produced by MDA-MB-361 cells act as growth factor and cooperate with HER/ErbBs ligands to stimulate the growth and proliferation of these cells in vitro and in vivo. In the study presented in additional file 6:S3 ppt we show that overexpression of DCD in SK-BR-3, a HER2+ amplified cell line, increases tumor growth in the mammary fat pads of NUDE mice as observed after overexpression of DCD in MCF-7, which is HER2 non-amplified breast cancer cell line (Figure 6).

Major compulsory revisions:

1. This manuscript describes at first time the co-expression of DCD and DCD-SV in normal skin tissue and breast cancer cell lines. The DCD splice variant is identical in its nucleotide sequence to larger (DCD-SV-2) form of DCD in human placental tissue described by Motoyama and colleagues (Motoyama et al., Biochem Bioph Res Commun 357(4): 828-837, 2007). We also found that DCD-SV and DCD are expressed at 2:1 ratio in placenta (data not shown). We designed double-stranded oligonucleotide against different exon sequences of DCD and cloned them in three specific shRNA lentivirus vectors. We analyzed the expression of DCD and DCD-SV transcripts by PCR in MDA-MB-361 cell clones transfected with IBC-I, II and III vectors and found that ICB-I vector containing a sense 5'-CCG GTT GAT CCC ATG ATC TCT TCT-3' and anti-sense 5'-AAT TCA AAA ACC TAG ATC CCA AGA TCT CCA ACT CGA GTT GGA GAT CTT GGG ATC TAG GA-3' oligonucleotide sequence was able to block the expression of both DCD and DCD-SV transcripts. So, this vector was used for to known-down the DCD transcript expression in cell lines used in biological assays described in this manuscript. Thus, we cannot conclude whether DCD-SV has the same effects on the expression of HER/ErbB family of receptors and ligands from the analysis of data presented in this study.

2. Our previous studies using serial analysis of gene expression (SAGE) and immunohistochemistry in over 675 samples of breast tumors in different histological stages confirmed that DCD is overexpressed in less than 10% invasive breast carcinomas (Porter et al., 2003a, PNAS 100(19): 1091, 2003). In this study we have examined the levels and patterns of DCD expression by IHC in more than 600 tissue samples arrayed in tissue slides (TMA). The majority of samples were negative for expression of DCD. We identified a group of 26 samples (4.3%) with consistently DCD immune reactivity in <50% or >50% of tumor cells. This subgroup was classified into subgroups according to their clinical and pathological features. Statistically significant associations (p<0.05) were found between DCD reactivity >50% and the subgroups with either high histological grade or with HER2 score 3 (Table 1). No relationship with overall survival was found. These results are in line with the findings of previous studies (Porter et al., 2003a, PNAS 100(19): 1091, 2003). The presence of DCD in HER2+ and luminal patient subgroups was expected since we have confirmed by PCR analysis that a small number of luminal B breast cancer cell lines with amplified HER2, including MDA-MB-361, EVSA-T, BT-474, SUM-185, SUM-190 and other cell lines express DCD at high level (Figure 1 and table S2).

3. The CK expression is used to classify subtypes of breast cancers; cell lines derived from basal-like breast tumor express CK-5/6 whereas cell lines derived from breast tumor of luminal origin express CK-8/18 (Hoadley et al., BMC genomics 2007, 8:258). MDA-MB-361 is a luminal cell line (Neve et al., Cancer Cell 10: 515, 2006). We compared the expression of CK-5 and CK-18 (Fig 3) in tumor tissues from the MDA-MB-361 cells of pKLO clone and IBC-I clone with constitutive expression of shRNA to DCD. The expression of CK-5 did not change in these tumor cells. The cells of IBC-I clone expressed high levels of CK-18 implying that they acquired
a more differentiated phenotype. The high expression of CK-18 may be contributed to slow growth and high sensitivity to cytotoxic agents of IBC-I clone as compared to cells of pKLO clone. We have not investigated whether the expression of CK8/18 is modulated after the overexpression of DCD in other cell lines analyzed in this study.

The association of DCD and HER/ErbB gene family expression was also investigated the microarray data sets of 55 human breast cancer cell lines obtained from Cancer Cell Line Encyclopedia (Additional table S1 and Figure S1). We did not find statically association of EGFR and DCD expression using the mRNA expression of microarray data. The transcriptional modulation of EGFR and its ligands by DCD has been investigated experimentally in five cell lines: MDA-MB-361, MCF-7, SKBR-3, HB4a and HB4a C5.2.

4. Our conclusions are based on the results from a genomic, biochemical and biological approaches wildly used to investigate gene-regulated cellular processes. This study aimed at investigation of the biological consequences of downregulating DCD expression in the MDA-MB-361, a human amplified-HER2+ breast cancer cell line and upregulating DCD gene in the MCF-7, a human HER2- breast cancer cell line and SK- BR-3, a human amplified-HER2+ breast cancer cell line. The global gene expression profiles in MDA-MB-361 control cells (pLKO group) and DCD shRNA expressing cells (IBC-I group) showed that knockdown of DCD has an impact on the expression levels of hundreds genes (208 down-regulated and 27 up-regulated), including the members of HER/ErbB gene family of receptor and ligands, which were further investigated. The biologic importance and molecular mechanism of action of HER/ErbB gene family at cell proliferation, resistance to cell death, and tumor growth processes are well known (Citri and Yarden, Nat Rev Mol Cell Biol 7:505, 2006). Down-regulation of DCD resulted in decreased levels of mRNA and protein and phosphorylation status (activation) of EGFR as well as phosphorylation of kinases Akt and MAPK, which are required to the mitogenic signaling pathway of EGFR/HER complexes. On contrary, overexpression of DCD resulted in increased expression and phosphorylation of EGFR. The c-MYC transcription factor, which controls the expression of numerous genes involved in metabolism, protein synthesis, and cell proliferation was also up-regulated in DCD expressing breast cancer cells or treated with rhDCD (Figure 5 and 6).

Many studies have confirmed the cell growth and survival activities of DCD after expressing of gene in tumor cells, but none is known about of resistance mechanism to cytotoxic agents (papers 6-13 of the reference list). Many successful therapies to tumors over-expressing EGFR and HER2 are effective at inhibiting overactive receptor tyrosine kinases or blocking extracellular portion of these membrane receptors; however long-term treatment ultimately leads to increased resistance by unknown mechanisms (Sergina et al. Nature 445: 437-441, 2007). A recent study published by Wilhelm and colleagues (Nature, Volume 509, page 582-587) has confirmed the role of DCD at cellular resistance to EGFR/ErbB inhibitors. In Wilhelm’s paper, Figure 5b displays a list of genes (biomarkers) that confer resistance against EGFR kinase inhibitors, in particular, Erlotinib and lapatinib. The analysis at protein and mRNA levels confirmed that DCD is in top protein list conferring cellular resistance to many tumor cell lines.

We have explored the pharmacological effects of combination of the trastuzumab, a humanized monoclonal antibody against HER2, and goat polyclonal antibodies against DCD in MDA-MB-361 breast cancer cell line. HER-2 specific antibodies prevent heterodimerization of HER2 with ligand-activated HER-family members. The treatment of mice with the anti-DCD antibodies or combination of trastuzumab caused a significant reduction of MDA-MB-361 xenografts in NUDE mice (Figure 7). Overall these results demonstrate that DCD autocrinally produced by cancer cells act as growth factor and cooperate with HER/ErbB receptor ligands to stimulate their growth and proliferation. Although there is as yet no clear evidence of how their signaling pathways operate together, our data suggest that DCD modulates EGFR/HER signaling network in HER2-driven breast cancer cell lines.
5. We have not shown the results of experiments xenograft growth of MCF-7-pcDNA and MCF-7-DCD cells stably expressing DCD in NUDE mice since a similar study was done by Monitto and colleagues and published time ago (Clin Can Res 10: 5862-5869, 2004). We have now shown the results of xenograft growth curves of control SK-BR-3-pcDNA and SK-BR-3 cells stably expressing DCD in the mammary fat pads of female NUDE mice (Figure S6).

Minor Essential revisions

1. The cut-points for DCD expression (RMA ≥4), EGFR (≥7), HER2 (RMA ≥8) and HER3 (RMA ≥9) expression were chosen after statistical analysis of entire groups (55 samples) and determination of the median RMA value of each data set. The groups of cell lines were subdivided in two groups: RMA lower (indicated as <) or RMA higher (indicated as ≥). These criteria are stated in the legend of table S1 and Figure S1.

2. The TNF doses used are normalized used in in vitro experimental assays for testing TNF cytotoxicity in human and mouse cell lines. In new version of manuscript we stated that only at highest dose TNF protected the cells. H₂O₂ induce apoptotic or necrotic cell death, depending on the concentration of the oxidant applied; low concentrations of H₂O₂ preferentially activated the caspase-dependent apoptotic pathway, while high concentrations of H₂O₂ induced apoptotic and necrotic cell death in a caspase-independent manner. It is well known that different cell lines are lower or rightly resistance from these genetically-regulated cell death programs (Circu ML and Aw TY; Free Radic Biol Med. 48:749-762. 2010). We have not investigated these mechanisms of resistance in the MDA-MB-361 cell line or its derivatives.

3. The MetaCore is a systems biology platform for functional enrichment, interaction analysis and network building of Omics data (thomsonreuters.com/metacore/). In the analyses in our studies were done as essentially described in the reference paper (Nikolsky et al., Cancer Res 68:9532-40, 2008). We have included additional file 7 that display the network legends, symbols and elements of figure 4.C.

4. We have made the corrections in the text and omitted NRG3 in the set of gene modulated as show the results of Figure 5A and 5G.

5. We have made the corrections on the x-axis number in Figure 5B and correctly described it in the text.

6. The concentrations of DCD, previously given in ng/ml, now have been correctly depicted in nM, as it appears on Figure 5C.

7. We have made the changes in the text and legend on Figure 4 to provide a comprehensive understanding of the data and functional interactions among genes up and down regulated in the Development_ERBB signaling canonical pathway map. The sentence now reads: “Betacellulin, amphiregulin, EGFR and c-Myc expression levels decreased in each of the three different DCD shRNA expressing cell pools compared to control pLKO. The text of legend is also describing the same. The additional file 7 shows the network legend and symbols and elements of ERBB the signaling pathway that is presented in the figure 4C.

8. We have made the corrections on the legend to Figure 2A-D and rewritten correctly in the text of the revised version of the manuscript.
9. We have added the micrographs showing the CK-5 expression in tumor cells of MDA-MB-361 cells of pKLO clone and IBC-I clone in the Figure 3. We have rewritten correctly the text and legend in the revised version of the manuscript.

10. We have made the corrections on Figure 4 and rewritten the legend of the heatmap describing the gene differently expressed.

11. We have discussed the issues raised along the answers to the questions 3 and 9 stated by this reviewer.