Author's response to reviews

Title: HEX expression and localization in normal mammary gland and breast carcinoma

Authors:

Cinzia Puppin (pupp.cin@tin.it)
Fabio Puglisi (fabio.puglisi@med.uniud.it)
Lucia Pellizzari (lpellizzari@mail.dstb.uniud.it)
Guidalberto Manfioletti (manfiole@univ.trieste.it)
Marta Pestrin (martute@libero.it)
Maura Pandolfi (maura.pandolfi@tiscali.net)
Andrea Piga (andrea.piga@med.uniud.it)
Carla Di Loreto (carla.diloreto@uniud.it)
Giuseppe Damante (gdamante@mail.dstb.uniud.it)

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Author's response to reviews: see over
To: The Editor-in-Chief of BMC Cancer

From: Prof. Giuseppe Damante
Dipartimento di Scienze e Tecnologie Biomediche
Piazzale Kolbe 1,
33100 Udine - Italy-

Udine, 22-3-2006

Dear Editor,

Enclosed here, please, you find the revised version of the manuscript entitled "HEX expression and localization in normal mammary gland and breast carcinoma" by Cinzia Puppin et al., (Manuscript n°: 3059545768811718) that we would like to submit for publication to BMC Cancer.

All criticisms raised by Referees have been addressed. Additional experiments have been performed and new data are shown. Detailed lists of how Referee’s criticisms have been addressed follow in the next pages.

We hope that in this version the present paper is suitable for publication in BMC Cancer.

Send, please, correspondence to:

Prof. Giuseppe Damante
Dipartimento di Scienze e Tecnologie Biomediche
Piazzale Kolbe 1 - 33100 Udine - ITALY
Tel: +39 0432 494285/374
Fax: +39 0432 494379
E-mail: gdamante@mail.dstb.uniud.it

Sincerely yours,
Prof. Giuseppe Damante
Reply to Referee 1 (Nancy Carrasco).

Major points

1. A table summarizing the characteristics of tumour samples has been included (Table 1).

2. The contradiction pointed out by the Referee has been addressed in the Discussion section (Page 11, lines 4-15).

3. Controls of immunohistochemical experiments are indicated in the Materials and methods section (Page 4, lines 5-7 from the bottom).

4. According to the Referee observation, although a statistically significant correlation was observed between Hex nuclear localization and estrogen receptor status, we agree that the finding has a weak significance because of the very low cell percentage showing the nuclear HEX localization. Therefore, we decided to delete this statement from the results section of the manuscript.

5. The major focus of experiments shown in Fig. 2 consists in demonstrating that the ATRA treatment increases HEX expression and induces a diffuse nuclear localization. Since the different immunohistochemical patterns observed between ATRA-treated and untreated cells, we decided to show the time-course of ATRA action by evaluating the percentage of cells with intense and diffuse nuclear staining, with respect to those with only nucleolar staining (see the revised Fig. 2). It is important to note that in the ATRA-untreated cells an intense nucleolar positivity is present in most cells (page 8, lines 6-15 from the bottom). Thus, since this positivity, Western blots after nuclear/cytoplasm fractionation could have provided misleading results. Our results may suggest that the nucleolar localization could represent a control mechanism for HEX activity (see the relative comment in the first paragraph of the Discussion section). In terms of HEX expression, immunohistochemical data are nicely complemented by the real-time PCR data. To reinforce the proposed link with NIS expression, time-courses of HEX localization and expression are shown in Fig. 2. The HEX induction after 4 and 8 hours of 1 µM of ATRA treatment is fully compatible with previously published data indicating that when MCF-7 cells are treated with1 µM of ATRA, the NIS mRNA increased is already evident after 6 hours and peaks after 12 hours (Kogai et al., Proc Natl acad Sci, 97, 2000). This comment has been introduced in the Discussion section (page 8, lines 6-15 from the bottom). We understand that several additional experiments are required to
firmly establish that the NIS gene is a direct target of HEX. This task, however, is beyond the aim of the present investigation and deserves “per se” a specific study. According to these statements, in the Conclusions section, we changed the sentence “Data indicating the HEX is able to increase the NIS promoter activity…” to “Data suggesting the HEX is able to increase the NIS promoter activity…” (Page 12, lines 9-10).

6. Immunohistochemical data indicating the difference between lactating and non-lactating breast tissues in terms of HEX localization have been included in the revised Figure 3 (described in the Results section, page 9, first 4 lines).

7. The effects of HDAC inhibitors NaB and TSA on HEX expression and nuclear localization have been assessed by immunohistochemistry and real-time PCR. Quantitative results are shown in the Figure 5 of the revised manuscript and described in the results section (page 9, lines 1-10 from the bottom). Either NaB or TSA increased HEX expression and induced a diffuse and intense nuclear localization. Since we have previously reported that MCF-7 treatment with NaB and TSA increase NIS expression, these new data reinforce the functional link between HEX expression/relocation and NIS expression.

8. The Discussion section has been significantly refocused. Several peripheral issues have been deleted and comments about the new results obtained have been included.

Minor points
The quality of English has been improved. All suggested changes have been included.
Reply to Referee 2 (Takahiko Kogai).

Major points

1. A table summarizing the characteristics of tumour samples has been included (Table 1).

2. In the previous version, Fig. 1 C described a case of ductal invasive carcinoma with scanty immunoreactive nuclei. We agree with the Referee that this could be confusing for the reader. Accordingly, we changed panel C of Fig. 1 with a case of ductal carcinoma without HEX nuclear expression, which is an example much more representative of the entire series.

3. We agree with the Referee that in the previous version the MCF-7 images of Fig. 1 E and 2A were not consistent. We have repeated several times the MCF-7 staining with HEX antibody without pretreatments. Always the staining was very evident in the nucleoli, with a very weak staining in the remaining part of the nucleus. Often this latter diffuse weak staining of the nucleus is barely detectable. Thus, we have changed panel E of Fig. 1, including a more representative image of the HEX expression/localization in untreated MCF-7 cells.

4. Five cases of non-lactating breast tissues were compared to three cases of lactating breast tissues, in the same experiment. No significant differences were observed among the different cases of lactating breast. This is now specified in the Results section (page 9, lines 1-4). In the revised Figure 3, images of immunohistochemistry on lactating and non-lactating breast tissues have been included.

5. As suggested by the Referee, to avoid squelching effects, the empty vector (containing the CMV promoter) has been cotransfected with the reduced amount of HEX or PAX8 expression vector. The efficiency of transfection has been normalized by a second reporter gene: the βGAL gene, whose transcription is driven by the CMV promoter. These details of the transfection procedure are now described in the Materials and methods section at the paragraph “Cell transfection” (page 6, lines 7-17). The CMV-LUC was never used. Its mentioning in the previous version was simply a typing error. The number of samples tested is now indicated in the figure legend. The significance of differences is now indicated in the Results section (page 9, lines 11-13).
6. As explained in the reply of criticism n° 3, the HEX expression in MCF-7 is low (in basal conditions) and mostly located in the nucleolus. Also in the tumour specimens a nucleolar positivity is evident (see Fig. 1, panel B). Thus, with the change of panel E of Fig. 1 (MCF-7 in basal conditions), is evident that the HEX expression/localization of MCF-7 cells recapitulates, at least partially, what observed in tumour specimens.

**Minor points**

1. Word spacing has been fixed.

2. The suggested reference has been cited in the Discussion section (Reference n° 36).

**Discretionary revisions**

Since the strong nucleolar positivity of MCF-7 cells when untreated, we prefer immunohistochemistry with respect to Western blot.

It was a good suggestion. In our hands, however, the efficiency of transfection of MCF-7 cells is very low.

In our opinion, without any experimental evidence (such as gel-retardation assay), comments on potential cis-element(s) that could explain the HEX action have a very limited significance.
Reply to Referee 3 (Rossella Elisei).

Minor points

1. A brief comment on the potential role of HEX in the control of NIS in thyroid cells has been included (page 11, lines 8-11 from the bottom). Without performing specific experiments (i.e. gel retardation assays and others), we believe that comments on potential details of the functional relationship between HEX and NIS promoter could be not useful for the reader. Because the comments of another Referee, the association between estrogen receptor status and nuclear HEX has been removed.

2. We agree that the number of analyzed tissues is not very high. However, in the case of breast tumours we believe that the casistic is sufficient to indicate a clear difference with respect to normal tissues. With regard to relationships with other characteristic of the tumour specimens, we simply reported it, without a particular focus on it. In the case of lactating tissues, it is extremely difficult to recruit them. All three lactating tissues showed an important difference with respect to non-lactating tissues, without significant differences among them. This is now stated in the Results section (page 9, lines 1-4).

3. The method used to calculate the mRNA levels after real-time RT PCR has been included (page 6, lines 1-2).