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EDITORIAL

Strict rules are needed for validation of G-protein-coupled receptor immunohistochemical studies in human tissues

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G-protein-coupled receptors (GPCR) constitute one of the largest families of cell surface proteins. Not only do they regulate, after activation by hormones and neurotransmitters, a considerable part of the physiological processes in the body, but they also represent a major class of drug targets and thus play an increasingly important role in medicine. Indeed, more than 30 % of the clinically marketed drugs do act through GPCR and are therapeutically successful in a large spectrum of human diseases. It is, therefore, evident that increasing information is needed on the expression and precise distribution of the GPCR, particularly in normal and diseased human tissues. The density of GPCR is usually considerably lower than that of other cellular parameters, such as that for hormones for instance, sometimes challenging their detection with current methods.

Human tissue samples are complex as they usually include many distinct cell types and compartments (epithelial cells, endocrine cells, nerves, vessels, immune cells, etc.,). Therefore, it is mandatory to use morphological methods for an accurate identification of the cell types that do indeed express the GPCR. Various methods exist to measure and localize these receptors morphologically, including, next to receptor mRNA detection with in situ hybridization, the receptor protein localization with in vitro radioligand binding using receptor autoradiography (ARG; gold standard), or immunohistochemistry (IHC) with adequate antibodies. IHC has become very popular because it is an easy-to-perform morphological method with excellent resolution and other advantages: Only formalin-fixed tissues are needed, instead of fresh-frozen material, and the number of available commercial and non-commercial antibodies is rapidly increasing. The consequence is a plethora of papers describing IHC findings for GPCR in human tissues. Unfortunately, several of these published papers show often questionable data due to the use of poorly validated antibodies and/or protocols [1–4]. If we do not want to be overwhelmed by poorly relevant publications on GPCR-IHC in the near future and by further controversial discussions in the IHC literature, it is urgent to react and correct this unsatisfactory tendency. It will be crucial for scientific journals that regularly publish IHC studies to introduce recommendations or even clear rules which would have to be strictly followed by authors, who otherwise would incur the risk that the submitted paper will not be evaluated. This initiative has recently been taken by Endocrinology [5]. But it is evident that the adherence to such a strategy by all journals reporting IHC data would be beneficial. Valuable in this regard is also a set of recommendations for the validation of IHC assays in diagnostic settings that have recently been published by the College of American Pathologists [6].

What is needed, at first, is a rather basic information, namely a full description of the antibody characteristics (antibody name, manufacturer, code number, antigen sequence if known, species raised in, monoclonal/polyclonal, clone designation if applicable, lot number for polyclonal antibodies). It should further be confirmed that the antibody was tested successfully in cell lines expressing the receptor while negative controls in cell lines devoid of receptors and/or in wild type/knock-out mice should also be provided [1, 2]. Preferably, such data should be available for both fresh frozen and corresponding formalin-fixed materials.

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This preliminary validation of a GPCR antibody does not warrant its specificity in human tissues. The antibody needs further to be tested in human tissues as follows:

1 One should take care to identify and use adequate control tissues—as far as they are known—for the antibody to be validated, i.e., choose positive controls containing a known established human target tissue expressing the receptor of interest. Adjacent non-target tissue should clearly not react, i.e., "stain negatively." Antigen pre-absorption tests, such as negative controls with an excess of antigenic peptide (if available) should also be provided in adjacent sections of the same tissue samples. Various antigen retrieval methods as well as different antibody concentrations should be applied in initial testing. The results should be carefully evaluated to obtain an optimal protocol. Once established, any further tests should strictly adhere to this protocol. Precaution should be taken with pancreatic islets when they are used as positive controls for validation of hormone receptors—as commercial providers often do—because the islets have been shown to be occasionally "immunostained" non-specifically [7].
2 One should carefully check the cellular localization of the immunohistochemical reaction product: by definition, GPCR are cell surface receptors with seven transmembrane spanning domains. Therefore, under normal conditions, we expect to see an immunoreactivity localized at the cell membrane. This is at difference with other receptor families located per definition in the cytoplasm and/or in the nucleus, such as the androgen receptors [8]. For GPCR, therefore, a diffuse cytoplasmic IHC staining is a doubtful and most likely non-specific result which is often, erroneously, interpreted as specific staining of internalized receptors. Surely, GPCR can be internalized. Internalization is even part of the physiological mechanism of action for many GPCR; a specific condition necessary for this phenomenon to occur is usually an acute receptor stimulation by agonist treatment. It should be understood that internalized receptors have a very particular intracellular distribution, as they are usually internalized in circumscript endosomes; thus, they are not diffusely distributed in the cytoplasm [9, 10]. To precisely evaluate the cellular localization of receptors, an illustration at high magnification is, therefore, required. Often authors do not take advantage of the high resolution of IHC but rather prefer to publish overview pictures of low magnification that sometimes mask a poor quality of the immunostaining.
3 One should provide a western blot for the antibody used in the same human tissues that were found IHC positive. Such a blot should confirm the identity of the receptor detected by IHC by showing a single specific band of the expected molecular weight.
4 One should compare the IHC-based data with data obtained by use of another morphological receptor-measuring method (ARG; in situ hybridization) in the same samples. While of fundamental importance, such a specificity test, of course, involves considerable additional work [11] and is, therefore, often omitted.
5 Ideally, one should compare the obtained GPCR-IHC data with the localization of the same GPCR using another antibody recognizing a different epitope of the receptor when such a well-validated antibody is available.
6 It is true for every IHC study that an optimal formalin-fixation of the human tissue samples, according to the standard rules of surgical pathology, is mandatory [6].

Putting the above-mentioned tests into practice will likely prevent controversies and reduce experimental discrepancies. Otherwise, unnecessary repeats and reports of IHC experiments with no clear conclusion will be the consequence, as recently shown by examples in the field of receptors for somatostatin and GLP1 [1, 2, 12, 13]. Implementation of the above-mentioned tests would, therefore, not only considerably add to the quality of published IHC papers, but also help in reproducing important new data and permitting science to progress on a safer track.

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