IN VITRO EVALUATION OF MOLECULAR TUMOR TARGETS IN NUCLEAR MEDICINE: IMMUNOHISTOCHEMISTRY IS ONE OPTION, BUT UNDER WHICH CONDITIONS?

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ABSTRACT

The identification of new molecular targets for diagnostic and therapeutic applications using *in vitro* methods is an important challenge in nuclear medicine. One of the methods is immunohistochemistry, increasingly popular due to its easy-to-perform procedure. This review presents the case for conducting receptor immunohistochemistry to evaluate potential molecular targets in human tumor tissue sections. The focus is on the immunohistochemistry of G-protein-coupled receptors (GPCR), one of the largest families of cell surface proteins, representing a major class of drug targets and thus playing an important role in nuclear medicine. The review identifies common pitfalls and challenges, and provides guidelines in performing such immunohistochemical studies. An appropriate validation of target is a prerequisite for developing robust and informative new molecular probes.
The discovery of novel molecular tumor targets is of key importance for nuclear medicine; for a successful tumor imaging and targeted tumor radiotherapy in patients, nuclear medicine needs to rely on an adequate identification of specific tumor targets with \textit{in vitro} methods in large series of human tumor samples. Such a specific tumor target is for instance a receptor, to which only the natural ligand or specifically designed molecules will be able to bind with high affinity. A specific tumor target means also that preferably only tumor cells, and no other tissues or organs or compartments will express a sufficient amount of it. In the literature, there are numerous examples of receptors massively overexpressed in tumors but not in normal physiological tissues. Clearly, nuclear medicine is dependent on target specificity if it wants to fulfill its role to accurately diagnose tumors and safely treat tumors with as little side effects as possible.

The GPCR, one of the largest families of cell surface proteins, represent one of such ideal targets for nuclear medicine. 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 reliable information on the expression and precise distribution of the GPCR, particularly in normal and diseased human tissues is highly desirable. The cellular 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 \textit{in vitro} methods. The present article focusses on GPCR, even though other classes of targets (enzymes, kinases,
transporters, steroid hormones and growth factor receptors) would also have been subject of interest.

It is of prime importance that GPCR are evaluated in vitro first, in large series of human tumor samples, so that a clear information of their incidence and density can be given for these tissues. GPCR can be detected in vitro in human tissues by various methods. It should be mentioned that human tissue samples are not a homogeneous entity but are extremely complex, as they usually include many distinct cell types and compartments (epithelial cells, endocrine cells, nerves, vessels, immune cells, etc.) as well as pathological tissues (tumors, inflammatory and degenerative tissues, etc.). Therefore, it is mandatory to use morphological methods for an accurate identification of the cell types that do express GPCR. Various morphological methods exist to measure and localize these receptors, including a) receptor mRNA detection with in situ hybridization, b) receptor protein localization with in vitro radioligand binding using receptor autoradiography, a method based on the same principle than the in vivo imaging in patients, namely the binding to the tumor tissue of a radiolabeled analog showing specificity and high affinity to the investigated receptor, c) immunohistochemistry with specific antibodies, the subject of the present commentary.

Immunohistochemistry has become very popular because it is an easy-to-perform morphological method: it has an excellent resolution at the cellular level; it can be performed on readily-available formalin fixed tissues rather than on the more difficult to obtain fresh-frozen material; the number of available commercial and non-commercial antibodies is rapidly increasing; it is generally considered easy to interpret immunohistochemistry data (see below for caveats, however). The consequence is a
plethora of papers describing immunohistochemistry findings for GPCR in human tissues. Unfortunately, several of the published papers show often questionable data due to the use of poorly validated antibodies and/or protocols (1,2). Several papers have therefore recently documented the necessity and relevance of well-designed immunohistochemical protocols and choice of correctly characterized antibodies (3-6). These authors also suggest that, in order to prevent poorly relevant publications on GPCR immunohistochemistry and subsequent further controversial discussions, the scientists publishing immunohistochemistry studies should follow recommendations or even clear rules (3,4,7). An initiative to introduce such recommendations has recently been taken by the journal Endocrinology (8); the adherence to such a strategy by all journals reporting immunohistochemical data would be beneficial. Valuable in this regard is also a set of recommendations for the validation of immunohistochemical assays in diagnostic settings, that have recently been published by the College of American Pathologists (9). As nuclear medicine is basically dependent on a precise definition and identification of receptor targets in vitro, basic scientists in the nuclear medicine field should be aware of this initiative.

What is necessary to make an immunohistochemistry investigation convincing? At first, a rather basic information is needed, 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 has been 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.
(3,4,10,11). Preferably, such data should be available for both, fresh frozen and corresponding formalin-fixed materials.

A validation of a GPCR antibody in cell lines does not necessarily 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 - to validate the chosen antibody, 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, i.e. negative controls, performed in presence of 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. Also frozen tissues may be tested. In many instances, immunohistochemistry works on frozen tissues; this is of importance since there are targets that need to be analyzed on frozen tissues because they do not survive formalin fixation. All 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 (12).

2) For GPCR, it is not sufficient to simply show the presence of the immunoreactive product in the cell, but it is essential to document where in the cell the
immunohistochemical reaction product is localized: by definition, GPCR are cell surface receptors with 7 transmembrane spanning domains. Therefore, under normal conditions, we expect to see an immunoreactivity localized at the cell membrane (10,11,13-15). A typical example of the somatostatin receptor sst2 expressed in a neuroendocrine tumor is shown in Fig. 1A. This membrane-bound localization of such a GPCR is at difference with the cellular location for other receptor families, such as for instance the androgen receptors (16) located per definition in the cytoplasm and/or in the nucleus. For GPCR, a diffuse cytoplasmic immunohistochemical staining is a doubtful result and most likely a non-specific staining which is often, erroneously, interpreted as specific staining demonstrating internalized receptors. Surely, GPCR can be actively internalized from the cell membrane into the cell. Internalization is even part of the physiological mechanism of action for many GPCR; a condition necessary for this phenomenon to occur is usually an acute receptor stimulation by agonist treatment that triggers the internalization of the receptor-ligand complex. It should be understood that internalized receptors have a particular intracellular distribution, as they are usually internalized in circumscript endosomes; thus, they are not diffusely distributed in the cytoplasm (17,18). Fig. 1B shows an example of internalized sst2 receptors in a neuroendocrine tumor from a patient that received an infusion of the somatostatin analog octreotide during the surgical removal of his tumor. As documented in both examples of Fig. 1, an illustration at high magnification is required, to precisely evaluate the cellular localization of receptors. Often authors do not take advantage of the high resolution of immunohistochemistry but limit themselves to publish overview pictures of low magnification that sometimes mask a poor quality of the immunostaining.
3) One should provide a Western blot with the used antibody in the same human tissues that were found to be immunohistochemically positive. Such a blot should confirm the identity of the receptor detected by immunohistochemistry by showing a single specific band of the expected molecular weight (13,15,17).

4) One should compare the immunohistochemistry-based data with data obtained with another morphological receptor-measuring method (in vitro receptor autoradiography; in situ hybridization) in the same samples. While of fundamental importance, such a specificity test, of course, involves considerable additional work (13,15,19) and is therefore often omitted. It is, however, very important to confirm the immunohistochemistry results with alternative methods such as receptor binding since the epitope identified by immunohistochemistry does not necessarily correspond to the binding site of the radioligand. In vitro receptor autoradiography is particularly suitable to analyze targets for nuclear medicine, as it is based on the high affinity binding of a radiolabeled compound to the receptor, and represents therefore the corresponding in vitro method to the in vivo imaging.

5) Ideally, one should compare the obtained GPCR immunohistochemical data with the immuno-localization of the same GPCR using another antibody recognizing a different epitope of the receptor, when such a well validated antibody is available (19).

6) It is true for every immunohistochemistry study that an optimal formalin-fixation of the human tissue samples, according to the standard rules of surgical pathology, is mandatory (9).
In consideration of the enormous amount of future drug targets and disease biomarkers (20-22) it is particularly relevant to use adequate in vitro methods for tumor target definition. Putting most of the above-mentioned recommendations into practice will likely prevent unnecessary controversies and reduce experimental discrepancies, as recently shown by examples in the field of receptors for somatostatin, chemokines and glucagon-like peptide 1 (3,4,10,11,23,24). Implementation of the above-mentioned tests would therefore not only considerably add to the quality of published immunohistochemistry papers, but also help in reproducing important new data and permitting a more straightforward progress of science. In particular, an accurate in vitro target definition is crucial for clinical nuclear medicine, by reducing the number of unnecessary clinical trials on poorly defined targets.

**Bullet points emphasizing the “noteworthy” key messages.**

1. Preventing “poorly relevant publications on GPCR-immunohistochemistry, scientists publishing immunohistochemistry studies should follow recommendations or even clear rules.” (p.4)

2. “A validation of a GPCR antibody in cell lines does not necessarily warrant its specificity in human tissues.” This statement if followed by a list of six criteria that can be used to validate an antibody in human tissues. (p.5)

3. “For GPCR, it is not sufficient to simply show the presence of the immunoreactive product in the cell, but it is essential to document where in the cell the immunohistochemical reaction product is localized.” (p.6)

4. “Accurate in vitro target definition is crucial for clinical nuclear medicine, by reducing the number of unnecessary clinical trials on poorly defined targets.” (p.8)
REFERENCES


Fig. 1: A. Immunohistochemistry of sst$_2$ in a neuroendocrine tumor taken from an octreotide-naïve patient. Very strong membrane-bound somatostatin sst$_2$ receptor staining is seen in the tumor cells.

B: Immunohistochemistry of somatostatin sst$_2$ receptor in a neuroendocrine tumor from a patient who received an i.v. infusion of octreotide during the resection of the tumor. Due to the octreotide application, the receptors are not membrane-bound but have been internalized after octreotide binding and show a typical endosome-related intracellular pattern.