UDC: 616.33-006.6-097(496.5)
Original scientific paper

STANDARDIZATION AND OPTIMIZATION OF SILVER IN SITU HYBRIDIZATION (SISH) FOR HER-2 GENE ASSESSMENT IN ALBANIA

Siltana Zeneli[1,3], Majlinda Ikonomi[2,3], Mehdi Alimehmeti[2]

[1*]*Department of Biology, Faculty of Natural Sciences, University of Tirana, Tirana, Albania*
[2] *Department of Anatomic Pathology, University of Medicine, Tirana, lbania*
[3]*Laboratory of Anatomic Pathology, NUHC "Nene Tereza", Tirana, Albania*
[*]*Corresponding Author: e-mail: siltanazeneli@gmail.com*

Abstract

Her2/neu protein overexpression assessed by immunohistochemistry is the most commonly used method in routine practice because of the easy performance and low costs. Her2/neu for equivocal cases that can't be evaluated from only mmunohistochemistry, need to be assessed for gene amplification using in situ hybridization probes. Silver in Situ Hybridization is a quite novel assay that has recently been introduced in our laboratory practice in NUHC "Mother Teresa". It deserves more time and is more expensive compared to immunohistochemistry.
In this study, for the first time in Albania, we used silver in situ hybridization for Her2/neu status assessment on gastric carcinomas. Considering the importance of silver in situ hybridization in clinical diagnosis, beside keeping to strict protocols and using validated, reliable antibodies and diagnostics kits, we developed a series of optimization protocols and a personalized workflow for new and archived samples. Establishment of these protocols has increased the success of the assay in our laboratory, especially for samples more than 1 year old. However, the main issue about this study is reaching an effective result in economical restriction situations, where samples of current or recurrent cases need to be reviewed for HER2 status.

*Keywords:*Her2/neu; Immunohistochemistry; Silver in Situ Hybridization; gastric cancer.

1. Introduction

Studies have shown that HER2 is expressed approximately in 30% of patients with breast cancer and 17.7% of patients with gastric cancer.[1]The examination of HER2 protein expression by immunohistochemistry is being performed for more than a decade in Albania, mostly for breast cancer. Now it has improved from manual to automated method. Mostly, this examination was performed in one laboratory that was the central laboratory of immunohistochemistry. Actually, new centers are being developed. Sharing the methodology among the laboratories improves quality assurance for HER2 testing.
Silver in-situ hybridization (SISH) assay is a relatively new assay for evaluating Human Epidermal Growth Factor Receptor 2 (HER2) genomic amplification in the Albanian population. It's performed only in one laboratory which is the reference site, the Anatomic Pathology laboratory of NUHC "Mother Theresa" in Tirana.
Achieving good results is crucial and difficult when:

- you have to deal with ***samples from different laboratories***, which means different and sometimes unknown pre-analytical factors.
- ***archival tissue samples*** that have been archived for long periods. Optimization protocol is not yet well established for archival tissues. Although there is a recommended nominal protocol, it is not suited for formalin-fixed and paraffin-embedded samples that have been archived for long periods.
- ***economical restrictions*** which mean sometimes dealing with long periods of no possibility to perform SISH.

In this study we used for the first time in Albania Silver in Situ hybridization for Her2 status assessment on gastric carcinomas. We developed some optimizations to the recommended protocol and improved the decisional workflow for archival tissue samples. These protocols have increased the success of the assay in our laboratory, especially for samples more than 1-year-old.

The main issues about this study are:

- highlighting problems
- reaching effective results in economical restriction situations, where samples of current or recurrent cases need to be reviewed for HER2 status.

We think that our experience will add value to who intends to utilize SISH for HER2 assessment in gastric cancers.

2. Material and methods

In a cohort of fifty (n=50) tissue samples of breast and gastric cancer we used Ventana BenchMark XT for immunohistochemistry and Silver in Situ Hybridization, which is a fully automated system that offers faster and more efficient results avoiding human mistakes.

2.1*Recommended guidelines*

For optimal results **VENTANA** recommends:

- Tissue fixation in **10% neutral buffered formalin** (NBF)
- Fixation time at least **6h to 48h**
- Fixative amount used should be **15 to 20 times the volume** of tissue
- Fixation should be performed at **room temperature** (15-25°C)
- Sections should be cut approximately **3-4 µm thick**
- Sections should be mounted on **positively-charged glass slides**, super frost plus slides
- Slides should be **stained promptly**
- To **assure workflow** on Ventana platform
- Experience, qualification and **continuous information** of technical and medical staff.

2.2 *Applying recommended protocol guidelines*

Fifty (n=50) samples were examined forHER2/neu protein expression with immunohistochemical method. Of all samples twenty (n=20) samples were stained with HER2/SISH utilizing INFORM HER2 DNA Probe. At first, we applied the recommended strict protocol, kits and reagents on all samples for Her2 gene amplification.

- As ***internal*** control we used:
  - samples from external laboratories
  - Slide Xenograft Her2 Dual ISH 3-in-1

- As ***external*** control we sent our samples to:
  - Roche diagnostics (Hellas), Greece
  - Ventana, France

Results showed problematic results like:

- ❖ Absence of ISH signals
- ❖ Dust and red haze
- ❖ Weak counterstain
- ❖ Presence of nuclear bubbling

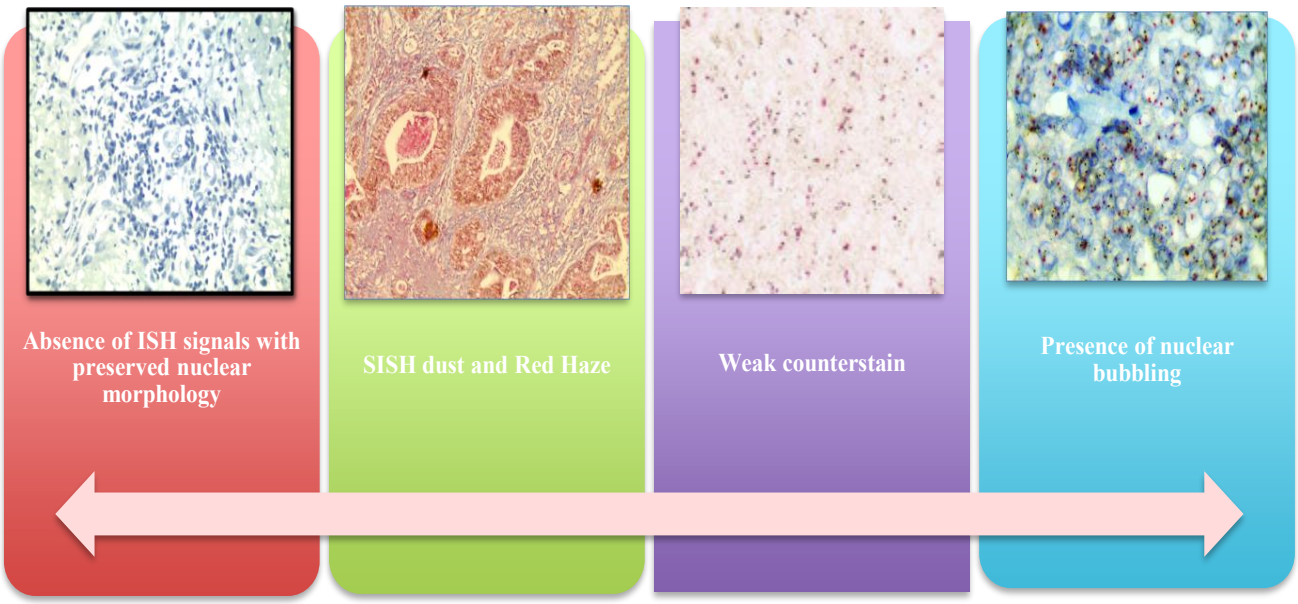

Figure 1. Results from recommended protocol to our samples

2.3 *Improving optimization protocols*

After first results, we tried to improve specimen and histopathological technique based on:

- fixation delay (Standardization starts in the Surgery Room)
- fixative type
- time in fixative
- reagents and conditions of dehydration
- paraffin impregnation
- conditions of slide drying and storage

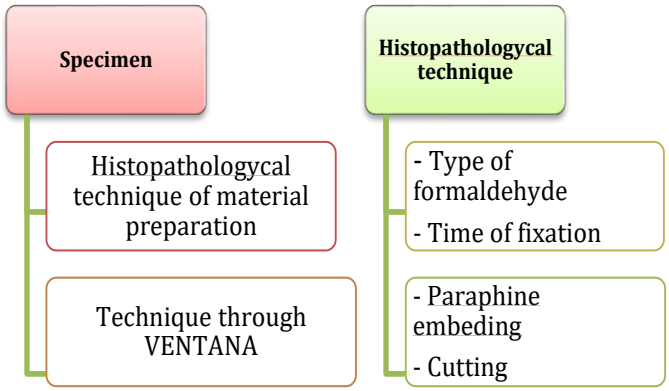

Figure 2. Protocol enhancing

3. Improved protocol changes

Based on our experience we developed a series of protocol changes that showed good results on SISH results.

Protocol step	**Recommended protocol**	**Optimized protocol**
Baking temperature	63°C	63°C
Baking time	20 mins	20 mins
Deparaffinization	72°C	72°C
Extended deparaffinization	Not selected	Not selected
Cell conditioning duration	3 cycles of CC2 at 86°C	3 cycles of **Reaction Buffer** at 86°C
	Mild CC2: 8 mins	Mild **RB:**12 mins
	Standard CC2: 12 mins	Standard **RB:** 16 mins
	Extended CC2: 8 mins	Extended **RB:**12 mins
ISH protease duration	ISH protease 2	**ISH protease 3**
	-16 mins	**-8 mins**
Denaturation time	20 mins	20 mins
Hybridization time	6 hours	6 hours
Stringency wash temperature	72°C	72°C
SISH multimer incubation time	32 mins	**36 mins**
Silver chromogen incubation time	4 mins	**8 mins**
Red ISH multimer incubation time	24 mins	**28 mins**
Red chromogen incubation time	8 mins	**12 mins**
Hematoxylin II incubation time	8 mins	12 mins
Bluing reagent incubation time	4 mins	8 mins

Table 1. Recommended protocol changes

After the evaluation of the results, optimal intervals for the key steps in both protocols were defined, according to which the remaining tissue samples were processed.

4. Discussions

Strict quality control standards are necessary for Her2 status assessment. There are many factors that can affect results: proper fixation, tissue processing, reagent quality, probe quality. Achieving good results with archived tissues is more difficult because of effects on fixation and accessibility of DNA. Optimal results were obtained with 24h of tissue fixation and 56°C overnight backing. Results on hybridization efficiency and signal quality were compared on the same group of samples with protocol improvements.

5. Conclusions

As a result of our experience, we can assess that:

- pre-analytical conditions can affect results of SISH assay
- age of samples and storage conditions were major factors in successful SISH assays
- samples that were less than 5-years-old, and archived in optimal conditions were successfully optimized.
- older samples, which were also archived off-site, have a higher frequency of unsuccessful optimizations.

Optimal signal quality was achieved after improving tissue handling, test standardization and experience in interpretations of Her2 results.

Nomenclature

IHC Immunohistochemistry
SISH Silver in Situ Hybridization
Her2 Human Epidermal growth factor Receptor 2

References

[1]. Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Ann Oncol 2008; 19:1523-9.

[2]. Hofmann M, Stoss O, Shi D, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology. 2008;52:797-805

[3]. Kim KC, Koh YW, Chang HM, et al. Evaluation of HER2 protein expression in gastric carcinomas: comparative analysis of 1,414 cases of whole-tissue sections and 595 cases of tissue microarrays. Ann Surg Oncol 2011;18:2833-40

[4]. Silver-Enhanced in Situ Hybridization as an Alternative to Fluorescence In Situ Hybridization for Assaying HER2 Amplification in Clinical Breast Cancer. Kyeongmee Park, Sehwan Han, Jung-Yeon Kim, Hyun-Jung Kim, Ji Eun Kwon, GeumheeGwak. J Breast Cancer. 2011 Dec; 14(4): 276–282.

[5]. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177-182

[6]. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, et al: American society of clinical oncology/college of americanpathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med. 2007, 131 (1): 18-43.

[7] YildizAktas IZ, Dabbs DJ, Bhargava R. The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma. *Mod*

[8] Pathol. 2012;**6**(8):1098–1105. doi: 10.1038/modpathol.2012.59. Faratian D, Graham A, Rae F, Thomas J. Rapid screening of tissue microarrays for Her-2 fluorescence in situ hybridization testing is an accurate, efficient and economic method of providing an entirely in situ hybridization-based Her-2 testing service. *Histopathology.* 2009; 6(4):428–43.