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SPECIAL ARTICLE

# Gene numerical imbalances in cytological specimens based on fluorescence/chromogenic *in situ* hybridization analysis

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## Summary

*Design and development of novel targeted therapeutic strategies is an innovation in handling patients with solid malignancies including breast, colon, lung, head & neck or even pancreatic and hepatocellular carcinoma. For a long time, immunohistocytochemistry (IHC/ICC) has been performed as a routine method in almost all labs for evaluating protein expression. Modern molecular approaches show that identification of specific structural and numerical imbalances regarding genes involved in signal transduction pathways provide important data to the oncologists. Alterations in molecules such as epidermal growth factor receptor (EGFR), HER2/neu, PTEN or Topoisomerase IIa affect the response rates to specific chemotherapeutic agents modifying also patients' prognostic rates. In situ hybridization (ISH) techniques based on fluorescence and chromogenic variants (FISH/CISH) or silver in situ hybridization (SISH) are applicable in both tissue and cell substrates. Concerning cytological specimens, FISH/CISH analysis appears to be a fast and very accurate method in estimating gene/chromosome ratios. In this paper, we sought to evaluate the usefulness of FISH/CISH analysis in cytological specimens, describing also the advantages and disadvantages of these methods from the technical point of view.*

**Key words:** chromogenic, cytology, fluorescence, *in situ* hybridization

## Introduction

Worldwide, cancer (derived from the ancient Greek word "ΚΑΡΚΙΝΟΣ", meaning a moving crab) is a major cause of death. According to almost recently published statistics, it is estimated that 1 out of 4 deaths in USA is due to a type of cancer [1]. Lung, prostate and colon carcinomas are responsible for most of cancer-related deaths in males. Concerning females, lung, breast and colon cancers are the leading causes of cancer-related deaths. Interestingly, pancreatic carcinoma is the 4th cause of death in both sexes, whereas liver (including intrahepatic bile ducts) and ovarian carcinomas represent almost equally the 5th cause of death in men and women [2].

Based on the molecular knowledge that has been achieved during the last 5 decades, cancer is not a simple disease, but a syndrome that involves a variety of gross chromosomal and specific gene alterations during the carcinogenetic process [3]. Concerning solid tumors, many functional and numerical imbalances that affect crucial molecular pathways such as cell cycle regulation, signaling transduction, apoptosis or angiogenesis have been identified and explained [4]. Aberrant gene expression, including oncogene upregulation combined with suppressor gene downregulation leads to cell cycle instability [5]. Genomic imbalances due to point mutations, polymorphisms, abnormal gene copy number, or structural chromosomal rearrangements and epigenetic modifications are detectable by different molecular techniques.

Modern pathology and cytology are closely relat-

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ed with molecular biology. Morphology under bright field microscopy should be combined with chromosome and gene abnormalities that boost a specific neoplastic or cancerous cell phenotype [6].

The aim of this article was to explore the role and value of *in situ* hybridization analysis (ISH) in cytological specimens regarding the novel targeted therapeutic strategies in patients with solid malignancies.

## Signaling pathways and targeted therapeutic strategies

Biochemical stability (known as homeostasis) between internal and external cell microenvironment regulates normal cell growth and proliferation that leads to tissue differentiation and organ development [7]. Because there are many different molecules that affect cell to cell interactions, such as growth factors, regulation of the signal transduction is a very critical process. Signal transduction includes many pathways that join the external cell environment with the nucleus as the final target of genomic functions. Most of the well studied pathways are based on a combination of protein families that act as mediators of specific signals from the membrane to the nucleus [8].

Growth factors and their receptors play a significant role in cell proliferation, adhesion, migration and differentiation [9]. The EGFR family includes 4 major transmembrane receptors: EGFR (HER1), c-erbB-2/neu (HER2), erbB-3 (HER3), and erbB-4 (HER4). Biochemically, the receptors consist of three major, separate domains: an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and a cytoplasmic tyrosine-kinase chain. Endocytosis of the complex stabilizes normally the communication between membrane activity and gene expression in the nucleus [10].

EGFR gene is located on chromosome 7 (7p12) and its product is a 170 KDa protein. Ligands, such as EGF or TGF-α, bind to the extracellular domain of the receptor and trigger a cataract of reactions, including dimerization and phosphorylation of the intracellular part and finally signal transduction to the nucleus is mediated by the involvement of RAS/RAF/MAPK proteins predominantly and via an alternative pathway (PI3/AKT/mTOR) [11]. In aggressive tumors, such as glioblastomas, EGFR gene amplification is correlated with shorter survival and resistance to radiotherapy [12]. Quite recently, novel targeted therapeutic strategies including anti-EGFR agents, such as monoclonal antibodies and small molecules, have been approved for the treatment in EGFR-dependent malignancies, including colon, lung and pancreatic carcinomas [13]. Although EGFR protein overexpression is observed in different proportions (25-80%) of colon adenocarcinomas, the crucial process for a successful therapeutic approach (response to chemotherapy and to novel targeted agents, survival benefits) remains the identification of specific gene deregulation mechanisms. Some studies have already suggested that there is an association between EGFR gene amplification and specific point mutations at exons 18-21 with the prognosis of the disease combined with absence of k-ras point mutations (wild type k-ras) [14-16].

The HER2/neu gene, also known as rat neu or c-erbB-2, is located on the long arm of chromosome 17 (17q12-21.32) and encodes a 185-KDa transmembrane glycoprotein which demonstrates tyrosine kinase activity. Signal transduction to the nucleus is mediated by ligand-binding induced stabilization of the receptor dimmers which is followed by receptor autophosphorylation and recruitment of specific SH-2 proteins [17]. The current pathway (HER2/PI3K/PTEN/mTOR) is targeted by different agents. Targeted therapeutic strategies include recombinant humanized monoclonal antibodies (i.e. trastuzumab) which bind to the extracellular domain of the receptor, preventing excessive signal transduction to the nucleus [18]. Quite recently, besides breast cancer, gastric adenocarcinoma is a target for the same monoclonal antibody application. HER2/neu gene amplification is the appropriate molecular substrate for this specific therapeutic approach in both of those types of adenocarcinomas, although there is a heterogeneity in gastric cancer regarding the gene signals compared to breast cancer homogeneity [19-21].

Vascular endothelial growth factor (VEGF) acts as a key mediator of angiogenesis in cancers of different origins. VEGF gene is a member of the PDGF/VEGF growth factor family and is located on chromosome 6 (6p12). Its protein product (VEGF A) is a glycosylated mitogen acting as an endothelial cell growth factor, promoter of cell migration, and inhibitor of apoptosis [22]. Normally, VEGF induces endothelial proliferation and increases vascular permeability. Deregulation of its upstream regulators, such as hypoxia inducible factor 1alpha (HIF-1a) –a transcription factor responsible for the regulation of oxygen homeostasis– leads to a tumor-associated angiogenesis by its overexpression [23]. HIF-1a gene is located on chromosome 14 (14q21-24) and under normal oxygen microenvironment von Hippel-Lindau (VHL) gene targets its protein product for rapid ubiquitination and degradation. In contrast, under hypoxic conditions, HIF-1α is activated through PI3 kinase - AKT and MAPK-ERK pathways, binding with its complementary factor HIF-1β to the promoters of genes that mediate glycolysis and angiogenesis,

such as VEGF [24]. Aberrant secretion of VEGF due to hypoxia, activation of oncogenes, and even EGFR or an abnormal hormonal activity leads to an uncontrolled binding to specific receptors such as VEGFR-1 or VEGFR-2. A cataract of reactions, including phosphorylation of intracellular tyrosine-kinase chains, leads to tumor angiogenesis characterized by a structurally and functionally abnormal vasculature. Anti-VEGF monoclonal antibodies (e.g. bevacizumab) are applied predominantly in patients with colon adenocarcinoma [25].

## FISH/CISH-SISH: description and comparison of the methods

Among molecular methods that identify specific genetic abnormalities in solid malignancies, ISH is a very popular technique in the majority of labs worldwide [26]. In 1969, three independent study groups introduced a novel process for the detection of specific DNA sequences [27]. The initial protocol was based on a radioactive (tritium) DNA labelling in proliferating cell populations. This was the first *in vivo* method for detecting DNA nucleotide chains. This method was entitled as "*in situ*" from the corresponding latin word meaning "in the original or true place (inside the nucleus)". In fact, ISH is the molecular method that localizes and detects specific DNA or RNA sequences based on radioactively, fluorescently or chromogenically labeled probes. ISH' evolution was assessed by Polak et al. [28]. Since then, modifications in ISH protocols have been made, but the philosophy of the method remains the same. Concerning the molecular procedure, formalin-fixed paraffin-embedded tissue sections or cytological specimens (FNA, fluid, intraoperative imprints) on conventionally fixed slides or on liquid based fixation are perfect substrates for ISH analysis. Membrane and cytoplasm lysis is the first stage for the detection of specific DNA/RNA sequences. In order to permeabilize the membranes, cells are treated by agents such as proteinases. Denaturation of double-stranded DNA is a critical step in this process. DNA denaturation based on specific conditions (heat/PH) is followed by probe annealing to the target sequence and finally to a hybrid formation. The final hybrid is a stable nucleotide sequence that can be visualized under bright-field or fluorescence microscopes based on the selected (chromogenic or fluorescent) labelling agents.

Before 2001, FISH was regarded as the gold standard method for detecting numerical imbalances, especially in malignancies that are involved in targeted therapeutic protocols (e.g. HER2 gene amplification in breast cancer) [29-31]. Introduction of CISH or its alternative SISH was an innovation in handling tissue or cytological specimens for ISH analysis [32]. CISH/SISH methods are based on an immunohistocytochemistry reaction which leads to genes and chromosomes visualized as scattered or in clusters signals labeled by chromogens (DAB, Methyl Green, Fast Red, and Silver). In contrast to FISH visualization, there is no need for fluorescence microscopes, only bright-field ones. Furthermore, slides treated by CISH/SISH techniques are permanently stored, like immunostained ones. FISH-treated slides are only temporally visualized due to the short life of UV effect in the corresponding fluorescence labeled probes.

Many studies have shown that FISH/CISH-SISH methods demonstrate a high concordance in evaluating numerical imbalances including HER2/neu and EGFR genes [33-36]. In fact, interpretation of gene/chromosome signals in those methods is mediated by different guidelines (Table 1). FISH with double, such as HER2/CEP17, or triple HER2/TopoIIa/CEP17 colored probes are interpreted as a ratio between overall gene to overall centromeric spots in 20 to 40 intact, non overlapping nuclei. Based on the extracted ratio, the genetic abnormality is characterized by the terms gene amplification, gene deletion, and/or aneuploidy/polysomy. Cases that demonstrate a ratio of 1 with two gene and two centromeric signals are characterized as normal (diploid), whereas a ratio of 1 with > 2 genes and centromeric spots correlate with polysomy without gene amplification. Cases with small or large gene clusters are characterized as gene amplified correlated with or without aneuploidy/polysomy (depended on the number of centromeric signals) [37].

At the onset of the method, the majority of CISH/SISH protocols were based on two separate probes (one for a gene and one for the corresponding chromosome centromere). Because all of the gene and chromosome signals should be visualized by the same chromogen (i.e. DAB), the estimation of a ratio was not possible.

**Table 1.** FISH & CISH-SISH interpretation criteria (for HER2 & EGFR genes)

| | *FISH CISH/SISH* (Gene/Chr ratio)* | *CISH/SISH** (Gene signals only)* |
|---|---|---|
| Normal (diploid) | 1 | 2 (2) |
| Non amplified | <1.8 | <4 (<5) |
| Low amplification (equivocal) | 18-2.2 | 4-6 (6-10) |
| High amplification (& clusters) | >2.2 | >6 (>10) |
| Deletion (or loss of one allele) | <0.8 | 1 (1) |

*According to ASCO/CAP criteria (Am J Clin Pathol 2009; 131: 490-497) including double color CISH/SISH kit

**CISH-SISH criteria provided by manufacturer's protocols (in parenthesis)

**Table 2.** FISH & CISH-SISH characteristics (advantages/disadvantages)

| *Characteristics* | *FISH* | *CISH/SISH* |
|---|---|---|
| Microscopy evaluation | Specific (fluorescence)<br>Increased magnification (60×) | Conventional bright field low magnification 40× |
| Visualization of signals | Accurate, fluorescence | Accurate, chromogenic (DAB, silver) |
| Storage of the fixed slides | Temporal | Permanent |
| Identification of multiple different gene/ chromosome signals | Dual or multiple probes | Mainly one or two probes per slide |
| Interpretation of signals | Based on experience | Easy, fast |
| Correlation to morphology | Lack of cell features | Simultaneous tissue/cell morphology with genetic events (numerical imbalances) |
| Cost | Increased* | Decreased* |

*Based on a comparison between two methods including microscopy domains (FISH/conventional) and corresponding commercially available kits

So, in the cases that 6-10 scattered signals were identified, a pathologist or a cytologist should analyze the corresponding chromosome to exclude a possible polysomy. In such cases, it is not clear if a low gene amplification exists or the multiple signals represent a true polysomy. This is a technical disadvantage of CISH/SISH protocols that are based only on one chromogen. Recently, commercially available kits, including two different chromogens for simultaneous gene and chromosome identification (i.e. DAB, Methyl Green, Silver or Fast-Red) have been developed. This modification resolves this technical aspect and provides an accurate, secure and fast interpretation based on chromogenic-labeled techniques. It includes all the advantages of FISH with the elimination of the disadvantages of CISH/SISH methods [38]. Furthermore, CISH/SISH signal interpretation seems to be friendlier compared to FISH to pathologists/cytologists that are familiar with bright-field microscopy but not with fluorescence (Table 2).

## FISH-CISH/SISH applications in cytological specimens

Based on an increasing need for applying targeted therapies in subgroups of patients in a rational way (increased response rates to monoclonal antibodies, extended lifespan), oncologists demand molecular data derived from PCR or ISH analyses. Since the last two decades, ICC/IHC has been established as a basic method for evaluating oncogene /(HER2/neu, EGFR) protein expression, especially in breast and colon cancer [39-41] (Figure 1). Although ICC-IHC detects protein activity, there are some parameters that affect its accuracy. Selection of different clones that target specific epitopes, fixation factors, many different protocols and a wide subjectivity regarding the interpretation of the results are serious reasons that potentially modify the final oncologist's decision for applying chemotherapeutic agents in his/her patients [42-44]. Furthermore, although IHC/ICC analyses identify protein overexpression in critical molecules (HER2/EGFR), they do not provide data about the molecular mechanism that induces the corresponding expression.

FISH-CISH/SISH protocols are sensitive and specific, providing accurate molecular data regarding numerical or gross structural imbalances in tissue and cell substrates. The results are clearly demonstrated on the corresponding slides as gene/chromosome signals or clusters of them and the interpretation is easy, based on specific guidelines [45,46]. In fact, cytological specimens such as imprints, FNA fluid –especially liquid based– provide a better substrate for ISH analyses compared to the corresponding tissue specimens. This happens because nuclei in cytological slides are intact and widely spread, without extended overlapping. In contrast, a significant subset of nuclei on tissue specimens is overlapped and loses a part of its volume under microtome procedure (sections of 6 μm). This technical aspect affects partially the accuracy of the final interpretation regarding the corresponding gene/chromosome signals [19] (Table 3).

Since 2004, many studies have shown the alternativity and even superiority of cytological specimens in evaluating gene/chromosome numerical imbalances based on FISH-CISH/SISH techniques. The majority of them analyzed HER2/neu and EGFR genes in breast, colon, lung, pancreatic and hepatocellular carcinomas [47-55]. In some of them, the study groups propose specific ratios based on gene copies analyses in CISH protocols. Additionally, others analyzed estrogen receptor 1 (ESR-1) and Topoisomerase IIa genes on tissue or combined with cell specimens [56-58]. All of them provide significant data about the applicability of the methods in cytological specimens and also about modifications that are necessary for an optimal interpretation of the corresponding signals.

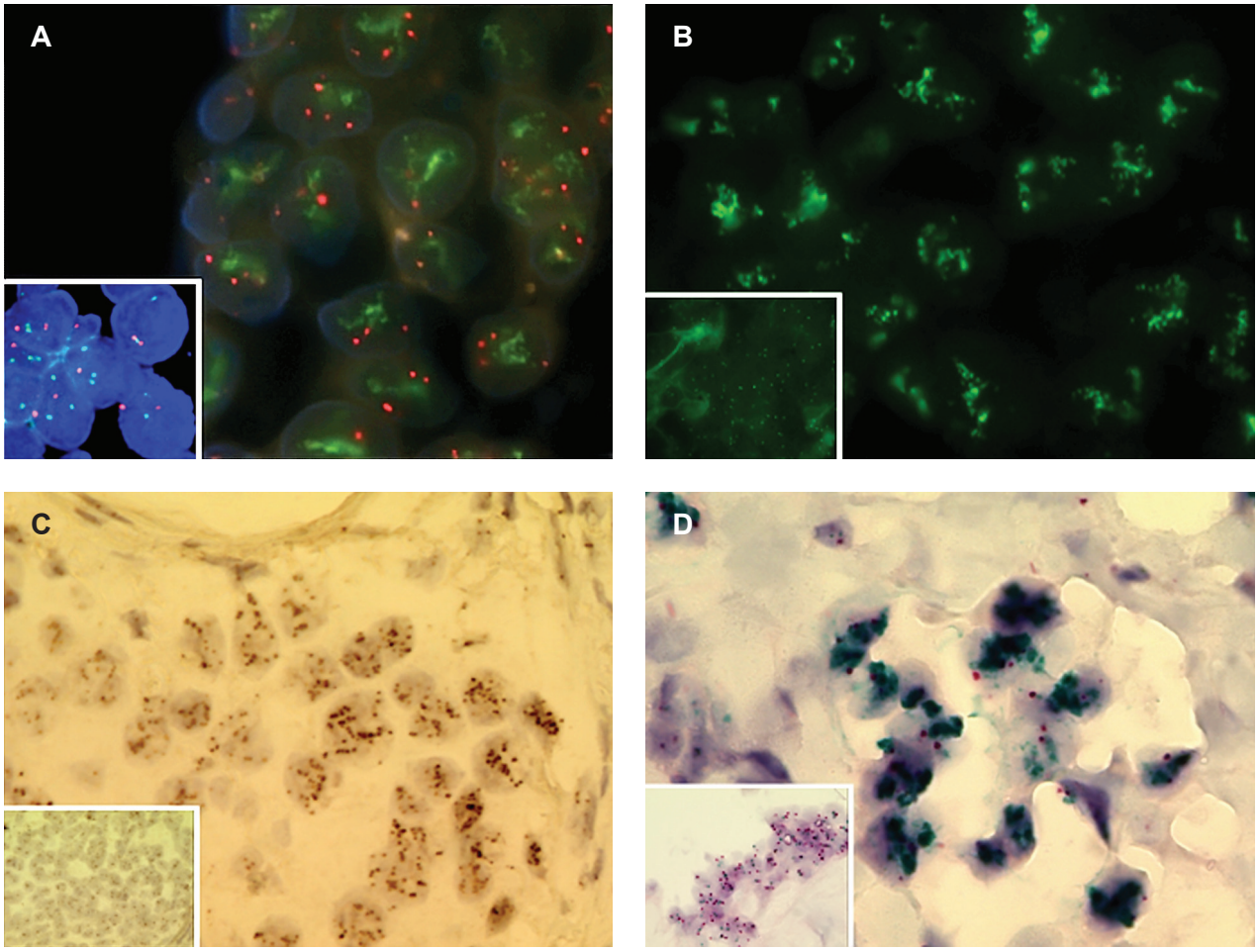


**Figure 1.** HER2/neu & EGFR FISH/CISH analyses in cytological and tissue specimens. **A:** breast adenocarcinoma with HER2/neu high gene amplification and focal chr 17 polysomy (3-4 red signals) on an FNA specimen (ThinPrep liquid based cytology). Note gene multiple copies and clusters (green signals) in non overlapping nuclei. **B:** EGFR high gene amplification in a case of colon adenocarcinoma (intraoperative imprint). Note clusters of EGFR gene copies spreading widely on the slide (green filter only). **C:** HER2/neu gene amplification on a tissue microarray core (breast adenocarcinoma) based on CISH. Note multiple gene clusters. **D:** Dual color CISH analysis in a HER2/neu high gene amplified breast adenocarcinoma case. Note the gene/CEP17 copies (green/red, respectively) on the same slide (original magnification: 60× for FISH, 40× for CISH). Inlet images represent normal (diploid) gene/chromosome ratios (=1) based on the same methods.

## Conclusions

Determination of gene/chromosome numerical imbalances by FISH and CISH-SISH protocols is a critical step for applying targeted therapeutic strategies in solid malignancies. These protocols are sensitive and specific in identifying numerical gene/chromosome imbalances. Based on an increasing experience regarding these ISH methods, we strongly suggest their performance in modern diagnostic and research Cytology. Every new study provides significant data for an optimal validation of these techniques and this is a basis for an improvement in relation with modern targeted therapeutic oncology.

**Table 3.** Comparison between tissue and cell specimens based on ISH analysis

| *Characteristics* | *Tissue (conventional/microarrays)* | *Cell samples (FNAs, imprints, fluids)* |
|---|---|---|
| Nuclear integrity | Limited (loss of a part due to microtome slicing) | Increased (nuclei are well preserved and spread widely) |
| Level of nuclear overlapping | Increased (due to 3D tissue construction) | Limited (especially due to liquid based fixation) |
| Interpretation of signals | Clear, but relatively decreased* | Statistically, increased number* |

*Based on studies that compare tissue and cytological specimens

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