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SHORT COMMUNICATION

Molecular assays in detecting EGFR gene aberrations: an updated HER2-dependent algorithm for interpreting gene signals; a short technical report

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Summary

Purpose: *Among oncogenes that have already been identified and cloned, Epidermal Growth Factor Receptor (EGFR) remains one of the most significant. Understanding its deregulation mechanisms improves critically patients' selection for personalized therapies based on modern molecular biology and oncology guidelines. Anti-EGFR targeted therapeutic strategies have been developed based on specific genetic profiles and applied in subgroups of patients suffering by solid cancers of different histogenetic origin. Detection of specific EGFR somatic mutations leads to tyrosine kinase inhibitors (TKIs) application in subsets of them. Concerning EGFR gene numerical imbalances, identification of pure gene amplification is critical for targeting the molecule via monoclonal antibodies (mAbs). In the current technical paper we demonstrate the main molecular methods applied in EGFR analyses focused also on new data in interpreting numerical imbalances based on ASCO/ACAP guidelines for HER2 in situ hybridization (ISH) clarifications.*

Key words: *epidermal growth factor, gene, in situ hybridization, polymerase chain reaction, receptors*

Introduction

The EGFR (other names include: ERBB ERBB1 HER1) gene is located on the short (p) arm of chromosome 7 at position 12 (cytogenetic chr band 7p12.1). The protein encoded by the corresponding gene acts as a transmembrane glycoprotein. It is a member of the v-erb-b2 erythroblastic leukemia viral oncogene (ErbB)/human epidermal receptor (HER) family of tyrosine kinase receptors, that includes also other three cell membrane tyrosine kinase receptors: HER2/c-neu (ERBB2), HER3 (ERBB3) and HER4 (ERBB4) [1]. All these members share mainly a common domain structure consisting of a large extracellular ligand-binding region, a single hydrophobic transmembrane bridge adjusting to an intracellular juxtamembrane (JM) region, a tyrosine kinase domain and finally a C terminal tail with multiple tyrosine residues acting as a regulatory region (with the exception of HER3 that lacks direct kinase activity) [2].

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Detecting specific genomic imbalances in solid and non-solid tumors is a crucial step for handling patients via targeted therapeutic strategies. Development of molecular methods including polymerase chain reaction (PCR) and *in situ* hybridization (ISH) drives the traditional histological profile of neoplasms to a novel genetic-based landscape, providing isolated genetic signatures in the corresponding patients [3]. EGFR mutations and also gene numerical imbalances are frequently detected in non small cell lung carcinoma (NSCLC), hepatocellular, pancreatic and also colon adenocarcinoma [4].

PCR assays

PCR was an outstanding revolution in nucleic acid analyses leading to an evolution in molecular diagnoses. In 1987, a group of investigators presented the first in vivo classical PCR technique [5]. PCR-based techniques are implemented in the vast majority of cancer analyses for detecting specific structural nucleotide alterations (mutations/insertions/deletions in genes' exons). Basically, the method - as a single PCR cycle - extends in three continuous steps. DNA denaturation is followed by primer hybridization or annealing and finally a new DNA double-strand is synthesized. Oligonucleotide primers, stable temperatures combined with DNA *Taq* polymerase enzyme are the main agents in every cycle. A number of approximately 10^7 to 10^{11} copies of the examined DNA region are produced after 30 to 50 repeated cycles [6]. Concerning m RNA as a template for PCR analyses, a reverse transcription polymerase chain reaction (RT-PCR) is implemented and a complementary DNA (c DNA) copy of the RNA is produced. The latter is used as a substrate for PCR multicycle analysis [7]. Real time (RT) PCR, including high-resolution melt (HRM) analysis, quantitative PCR (qPCR), sequencing PCR, Sanger-based or extended as massively parallel sequencing/next generation sequencing (NGS), and novel sophisticated variations including Ultra-Deep pyrosequencing (UDP), peptide nucleic acid (PNA)–mediated PCR clamping or enrichment PCR-UDP have been designed and applied in identifying EGFR mutations, including missense substitution, in-frame deletion and insertion [8-10]. There is a wide spectrum of commercially available assays and primers for diagnostic/research use. Interestingly, the enrichment PCR-UDP technique improved the level of mutation analysis by detecting rare mutations (with frequencies as low as 0.01%), especially in scarce tissue samples or those with small quantities even in heterogeneous samples [11].

ISH assays: the role of rational gene/chromosome signal number interpretation

In 1969, three independent study groups introduced a novel process for the detection of specific DNA sequences. The initial protocol was based on a radioactive (tritium) DNA labeling 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. [12]. 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 inprints) 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) labeling agents.

Based on an increasing need for applying targeted therapies in subgroups of patients in a rational way (increased response rates to mAβs) oncologists demand molecular data derived by ISH analyses. Since the last two decades, immunohistocytochemistry (IHC/ICC) has been established as a basic method for evaluating oncogene / (HER2/neu, EGFR) protein expression, especially in breast and colon cancer [13-15]. 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 targeted-chemotherapeutic agents in patients [16,17]. Furthermore, although IHC/ICC analyses iden-

tify protein overexpression in critical molecules (HER2/EGFR), they do not provide data about the molecular mechanism that induces the corresponding expression.

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) [18]. Introduction of CISH or its alternative SISH was an innovation in handling tissue or cytological specimens for ISH analysis [19]. CISH/SISH methods are based on an IHC-like reaction providing visualization of genes and chromosomes as scattered or in clusters signals labeled by chromogens (DAB, Methyl Green, Fast Red, and Silver). In contrast to FISH, there is no need for fluorescence microscopes, only bright-field ones. Furthermore, slides treated by CISH/SISH techniques are permanently stored, like the immunostained ones. FISH-treated slides are only temporarily visualized due to the short life of UV effect in the corresponding fluorescence labeled probes.

FISH/CISH-SISH methods demonstrate a high concordance in evaluating numerical imbalances including HER2/neu and EGFR genes. In fact, interpretation of gene/chromosome signals in these methods is mediated by official guidelines provided mainly by American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) editions (Table 1) [20]. According to these guidelines, FISH with double, such as EGFR/ CEN7, or triple HER2/TopoIIa/CEP17 colored probes are interpreted as a ratio between overall genes 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 normal for numerical imbalances, gene amplification, gene deletion, and/or aneuploidy/polysomy. Interestingly, in ASCO/ACAP 2013 updated recommendations, borderline cases with a HER2/CEP 17 ratio <2 but with an average HER2 copy number ≥6 signals per cell are diagnosed as positive for gene amplification. This is a very important approach and a progress for handling those cases by applying mAbs inducing the number of patients that may earn response and survival benefits. Based on the previous ASCO/CAP 2007 gene signals interpretation criteria, those cases were not considered eligible for anti-HER2 mAbs therapeutic regimens [20]. Identification of intra-carcinoma genomic heterogeneity due to rise of different cancerous clones is the explanation for this modification.

In conclusion, identification of specific gene deregulation mechanisms regarding growth factor receptors (ie EGFR, HER2) is critical for applying targeted therapeutic strategies. Based on ASCO/CAP 2013 updated criteria for HER2 gene signals evaluation, we suggest that these should also be adjusted similarly for EGFR ISH analyses interpretation eliminating borderline molecular diagnostic results.

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Table 1. Differences in interpretation of HER2/CEP 17 gene signals based on ASCO/CAP 2007/2013 criteria

Dual FISH/CISH/SISH (ASCO 2007 Guidelines): J Clin Pathol 2008;61:68-71	
1. Ratio HER2/Chr17 ≤1.7	No amplification
2. Ratio HER2/Chr17 1.8-2.2	Low amplification
3. Ratio HER2/Chr17 ≥2.3/clusters	High amplification
4. Ratio HER2/Chr17 =1	Chr 17 diploidy (if both HER2 & Chr17 = 2 copies) Chr 17 polysomy (if both HER2 & Chr 17 >2copies) Chr 17 monosomy (if only one Chr 17 copy in at least 30% of the examined cancerous nuclei)
Dual FISH/CISH/SISH (ASCO 2013 updated Guidelines): J Clin Oncol 2013;31:3997-4013	
1. Ratio HER2/CEP 17 ≥2.0	Positive for gene amplification with an average HER2 copy number <or>=4.0 signals per cell
2. Ratio HER2/CEP 17 <2.0	Positive for gene amplification with an average HER2copy number >=6.0 signals per cell
3. Ratio HER2/CEP 17 <2.0	Equivocal for gene amplification with an average HER2 copy number >=4.0 and <6.0 signals per cell
4. Ratio HER2/CEP 17 <2.0	Negative for gene amplification with an average HER2 copy number <4.0 signals per cell
5.	Undetermined

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