Detection of chromosome changes in pathology archives: an application of microwave-assisted fluorescence in situ hybridization to human carcinogenesis studies

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Pathology archives provide unique and abundant opportunities to investigate human carcinogenesis and identify potential targets for cancer therapy. Microwaving was introduced into various procedures used in histopathology two decades ago, although the precise mechanisms underlying its effectiveness in any of the procedures, including antigen retrieval, acceleration of fixation and nucleic acid hybridization, are not known. Since microwaving was first applied to fluorescence in situ hybridization (FISH), many pathologists and researchers have enjoyed the benefits of excellent preservation of histological structures as well as good retrieval of FISH signals by this method. Microwave-assisted fluorescence in situ hybridization (MW-FISH) has proved to be especially useful in retrospective investigations of tissues fixed and preserved for long periods of time, and the success rates in the randomly selected pathology archives have been greater (70–95%) than by the conventional protocol (≤40%). The MW-FISH protocol and current availability of human genome information together with information on a variety of other histopathological attributes have paved the way to exploration of specific, large-scale genomic changes in human tumor tissue, even in the incipient stage. In practice, this protocol is very useful for retrospective surveillance of amplifications in tumor tissue by using hundreds of bacterial artificial chromosome chromosome clones and many specimens in the form of a tissue microarray. Effective retrieval of specific genome-wide amplicon profiles from human tumors stored unawares in ordinary pathology laboratories would help to further stratify tumors so that individually tailored treatment strategies would become feasible in clinical settings.

Introduction

Fluorescence in situ hybridization (FISH) is an essential tool of diagnostic pathology for identification of amplifications and translocations of genomic components in human tumors (1), especially in hematological malignancies (2), childhood tumors (3) and sarcomas (4). FISH is also becoming a popular means of investigating common carcinomas having genomic amplification, aneusomy and copy number alterations (5–7). Reporting human epithelial growth factor receptor (HER2) amplification in breast cancer tissue as an important indicator of treatment and outcome predictor in individual cases is an essential task of pathologists in pathology laboratories everywhere (8–10). FISH combines molecular biology and histopathology techniques, and several pieces of equipment are necessary. Another obstacle is that not all pathology archives are ideal for FISH. Since extra effort is required to collect and handle samples that can be used for FISH in terms of fixation, embedding and storage, most retrospective studies have often been hampered by poorly preserved formalin-fixed paraffin-embedded tissues.

Abbreviations: BAC, bacterial artificial chromosome; CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization; FITC, fluorescein isothiocyanate; MW-FISH, microwave-assisted fluorescence in situ hybridization; SNP, single-nucleotide polymorphism; TMA, tissue microarray.

In this review, we describe a modified FISH protocol that includes intermittent microwave irradiation and was designed particularly for formalin-fixed paraffin-embedded tissues, and we describe its application to human carcinogenesis studies. We also mention genome-wide searching for amplicons in human tumors, which is facilitated significantly by the microwave-assisted fluorescence in situ hybridization (MW-FISH) technique.

Microwaving was introduced into the FISH procedure a decade ago. Prompted by the antigen retrieval effect of microwave irradiation (11), Wilkens et al. (12) demonstrated the effect of microwave treatment on RNA-FISH in paraffin-embedded tissues. Lu et al. (13) described a hybridization procedure that was accelerated by using microwave irradiation. In 1999, we and others developed microwave-assisted protocols for application to pathology archives (14–16). While Bull et al. used microwave irradiation as pretreatment, our protocol described below involves intermittent irradiation during the hybridization step. Increased efficiency and validity of signal detection with microwave irradiation had already been demonstrated in lymphocyte cytosmear preparations and cancer cell stamp preparations (15,17) (Figure 1). There is less slide-to-slide variation and preservation of histological structures and distinct intranuclear signals are maintained after hybridization. There are now several variations in MW-FISH protocols and standardization has been done adapting to each laboratory’s requirements (18).

Strategies

Merits of MW-FISH protocol when using tissue microarrays. Tissue microarrays (TMA) are popular means of gathering immunohistochemical information from thousands of small tissue samples at once. TMA is very useful for FISH analysis in terms of obtaining signals from many specimens at one time. Ordinary FISH procedures are applicable to some prospectively prepared and arrayed specimens, but ordinary FISH usually yields a low output when the arrayed specimens have different preservation histories. The MW-FISH protocol is especially powerful for the arrays consisting of specimens that have been fixed in various ways, stored and sometimes even neglected for a long time. Signals that were hardly retrievable by a conventional procedure or a company-recommended procedure have been successfully obtained in a variety of human tumors (19,20). Thus, experiences with MW-FISH show that it allows a wider selection of materials, for example, a larger piece of tissue that contains morphological heterogeneity and stromal cells, both of which are important in the characterization of human tumors. Actually, MW-FISH dramatically increases the efficiency of signal retrieval from each of hundreds of tissue specimens in arrays (from 40% by a conventional protocol to >95% by the MW-FISH protocol). If a histopathologist is a member of a research team, or if an investigator is familiar with morphological features and their significance in tumors, they can use ordinary hematoxylin-eosin array sections to monitor the validity of tumor portions. Depending on the purpose of the study, it is possible to use tissue array such as an early stage cancer tissue array (21), cancer in adenoma tissue array, boundary (premalignant and in situ) lesion array and array of tissue from multiple primary cancers in the same individual (22,23), or array from multiple regions of an extensive lesion, such as ulcerative colitis. It usually takes a long time to accumulate clinically important or infrequently encountered tissue samples in good enough condition for even ordinary FISH protocol to retrieve the signals. MW-FISH extends the horizon of the project by allowing archives of rare tumors and other materials to be used. In the sections below, we describe the basic procedures and strategy for retrieving signals and surveying genomic information in pathology archives.
In situ hybridization promoted by intermittent microwave irradiation. The steps in the protocol for FISH with intermittent microwave irradiation are shown in Figure 2. Sections of tissue blocks that have been stored in formalin for a long period of time (a month or longer) usually yield no signals when subjected to the ordinary FISH protocol without the use of microwave irradiation. Only a small improvement is achieved by protease treatment. Several pathologists, including the pathologists in our group, have applied various protocols besides the MW-FISH protocol and have had difficulty in obtaining stable results. However, in our experience, the MW-FISH protocol yields acceptable signals in >95% of such blocks, and an example of a block in which enhanced signals were obtained by this method is shown in Figure 3A versus B. The structure of the tissue under lower magnifications is informative for making morphological diagnosis (papillary cancer in this case, Figure 3C) (36), and the tumor cells in this tissue show distinct, countable signals under higher magnification. We do not know exactly why microwaving improves signal sensitivity, but scanning electron microscopy analysis of the formalin-fixed paraffin-embedded tissues has revealed a looser intranuclear matrix after microwave exposure (Figure 3E versus D).

The necessity of MW-FISH depends on the materials and their conditions. There have been a few reports (37,38), mainly in regard to brain tumor samples, of adequate FISH signals being obtained with commercially available protocols, but it is common knowledge among histotechnologists that commercial protocols that require stringent protease treatment are not readily feasible (14,39,40).

The most frequently used model of laboratory microwave generator in Japan is ‘M-77’ model (Boeckeler Instruments, Tucson, AZ; http://www.rmcproducts.com/, info@boeckeler.com). The success rate with this model and MW-FISH is better than that with other commercial protocols in the literature (41), and actually >90% for formalin-fixed tissues, irrespective of time they have been left in a non-alcohol fixative, and ~100% for alcohol-fixed tissues.

Repeated FISH application. The same as rehybridization of the probe on the membranes used for Southern or northern blotting, we reuse histological sections for multiple hybridizations with FISH probes by stripping the dyes used for the first hybridization. Repeated FISH with multicolored probes has been applied to FISH for metaphase (42). We inserted the microwave irradiation step in the procedure of stripping the first signal and rehybridization of the new probe in pathology archives. Using a virtual color system on a computer screen allowed us to present the results in multiple colors. The details of the procedure have been reported elsewhere (43). Rehybridization should be useful for multiple probrings of rare sections and/or small microscopic lesions. In our experiences, the repeated FISH procedure has only been successful when the MW-FISH protocol was used. The signals of the multiple loci obtained by MW-FISH have dosages (gain or loss) comparable with those obtained by copy number estimation algorithms such as the gene imbalance map (44) obtained from the SNP array data (45).

Double staining with immunohistochemistry. The microwave-assisted double staining with immunohistochemistry protocol allows localization of gene products, such as membrane proteins, and their genomic signals in the same cells. The details of the procedure are described elsewhere (46). Briefly, immunoreactivity in each cell is quantitated by scanning the density of the staining, and the dosimetric value is compared with the number of FISH signals in the corresponding cells. The genomic signal dosages and immunohistochemical staining of the product have always been well correlated (Figure 4) (46).

Evaluation of FISH images. Unlike evaluation of FISH image in cytological stamp specimens, evaluation of histological sections...
 involves only thin slices (6 μm thick) of the tissues. Thus, there may be miscounting in interpretation of the signals. The countings and interpretations of the number of signals per cell must take factors into consideration such as artificial loss due to the lack of the completeness of the nuclei observed and artificial gains as a result of counting the signals in overlapping cells. Observations are made with a fluorescence microscope (BX51, Olympus, Tokyo, Japan); the images are captured with a charged coupled device camera (PCO SENSICAM, Cooke Co., Auburn Hills, MI) and converted to the digital images with software MetaMorph (Universal Imaging Co., Downingtown, PA). The emission filters used for 4',6-diamidino-2-phenylindole (DAPI), FITC and Cy3 are XF1005 (365WB50), XF1042 (485DF15) and XF1045 (550DF15), respectively. The absorption filters used for DAPI, FITC and Cy3 are XF3002 (450DF65), XF3025 (615DF45) and XF3076 (695AF55), respectively. Spectrum Green (Abbott) is used in the same range as FITC, and Spectrum Orange (Abbott) is used in the range of Cy3. The amplified signals are qualitatively categorized into three types: a double-minute type, polyploidal type and homozygous staining region type. Examples of the marked gains are shown in Figure 5A–C.

Applications
The topics I briefly state here as potential applications of MW-FISH in cancer genome analysis in pathology archives have been investigated using conventional FISH technology (47,48), but many research groups have reported on the drawbacks of the ordinary FISH procedure, especially for pathology archives. The application of the MW-FISH protocol would promise to expand the possibilities to use wider varieties of materials, harvest greater genetic information and shorten the whole procedure (40).

Stepwise, non-random alterations in gastric carcinogenesis. Chromosome numerical abnormalities are one of the hallmarks of common human cancers, but there have been few anatomically defined stage-dependent description of such abnormalities based on pathology archives. The MW-FISH protocols combined with diligent pathological analysis of gastric cancers, including the intramucosal stage (early gastric cancer according to the Japanese classification system), have revealed that the numerical changes in chromosome are not random and usually involve chromosomes 1, 2 and 4 (17,21). They also revealed that most gastric cancers already exhibit aneuploidy in the very earliest stage.

Histopathological subtypes and chromosomal numerical abnormalities. The excellent preservation of histological morphology with MW-FISH signals by this modified FISH protocol enabled us to correlate chromosome numerical abnormality patterns with particular histological morphology. For example, well-differentiated adenocarcinoma is usually treated as a single entity, but its microscopic structure differs from place to place within the same tumor, and in some places, papillary and tubular structures coexist. Genomic signals obtained by MW-FISH protocol clearly identify the changes in situ in each of the parts that exhibit different morphological features (36). Furthermore, multiplicity and histological heterogeneity are areas in which MW-FISH protocol had advantages (22,23) in identifying cancer cell clones.
Early lung carcinogenesis and field cancerization. The fact that the MW-FISH method can be applied to lesions <5 mm in diameter enabled us to recognize that chromosomal numerical abnormalities are prevalent in the so-called precancerous lesions, such as adenomatous hyperplasia of the lung. A detailed description of the chromosomal abnormalities in early lung cancers and arguments about lung carcinogenesis are provided in a previous article (49). The concept of so-called ‘cancer in adenoma’ in the lung is consistent with the observation of mild chromosomal numerical aberrations in early adenomatous areas in the lung and severe chromosomal numerical changes within such areas (49). The specificity and the involvement of particular loci of the genome in the adenoma–carcinoma sequence of the lung require further investigation.

All these materials mentioned above (early stage cancer, histologically special subtype, multiple primary cancers and cancer in adenoma in lung) are usually often coincidentally encountered in a routine practice and retrospectively identified. Thus, retrieval of the signals from these fairly conditioned blocks is not easy. MW-FISH that had several advantages over the ordinary protocols is one of the essentials in the arsenal of the researchers in molecular pathology. We also admit grading the amplification signals may depend on completeness of the retrieval. We can monitor the dosage of signals at particular loci obtained by MW-FISH compared with the dosage obtained copy number estimation through CGH or SNP microarray.

Comparison with SNP array analysis. A gene imbalance analysis by CGH or SNP microarray analysis is often accompanied by FISH analysis in order to validate the areas of gain and loss in the genome. MW-FISH is the best means of validation in case SNP microarray gene imbalance study of human tumors is conducted. The amplified loci and their corresponding FISH images have been consistent, and that has been helpful in identifying uniparental disomy (24,45).

Expansion to genome-wide searches for amplicons in tumors in pathology archives. TMA-FISH surveillance may generate the amplification profile data by any hybridization methods for proper materials, but the prevalence and grade of amplification depend on the FISH method adopted when pathology archives are used. Comparison of the positive rate obtained using the MW-FISH protocol and the other FISH protocol in HER2-FISH tests, especially in immunohistochemistry 2+ cases, revealed the difference in amplification prevalence, implying that MW-FISH-TMA surveillance would be the most
reliable method of documenting the amplicon profile in human tumors. The profiles of amplicons appear to be vaguely specific for loci and organs. Some cancers contain multiple amplifying loci, and the recent discovery of multiple gene mutations within the same cancers has suggested that an unexpected high number of genes are mutated (50). As the investigator who commented on the article insightfully mentioned (51), other changes besides mutations play a role in carcinogenesis, not only as ‘passengers’ but also as ‘drivers’. We now know that amplification is also a ubiquitous event in human cancers (52), but we do not know whether the prevalence and loci of amplification may differ according to the FISH methods (MW-FISH or ordinary FISH) and whether that might lead to the differently biased views about the whole genomic alteration in human tumors.

We used 420 BAC clones, which covered every chromosome, to survey the amplified region and used them to validate improved retrieval of the signals of the loci. When the MW-FISH protocol was used, most of their signals became more distinct, especially in the archives that had been stored for a long time. Many of the same loci were found to be amplified in several different kinds of cancers, a finding that was compatible with the data reported in the recent literature (52,53). Several loci that are known to be useful clinically in a certain type of cancer are actually amplified in a wide variety of other cancers even in the early stage, and HER2 is not the only example (54). We would like to address a practical point in regard to interpretation of these amplified loci especially those detected only by MW-FISH method. The HER2 test (FISH detection kit for expression and DNA amplification of HER2) is now widely used in pathology laboratories worldwide, but the prevalence and evaluation of the signals are known to be influenced by protocols of FISH procedures (55). Our experience by MW-FISH extended this situation to almost all the BAC clones we tested. The MW-FISH procedure often increased the grade of amplification. MW-FISH may influence the interpretation of the amplified loci and ultimately the choice of therapy. Since the information selected from the profiles of several hundreds of amplicons in tumors should suggest potential target molecules to the clinical oncologist in the very near future, it is important to select the most reliable method for detection of unambiguous signals in the archives. MW-FISH procedures, such as the microwave-assisted antigen retrieval method, will become one of the routine modified FISH procedures used in pathology. Very recently, Bayani et al. (56) reviewed the TMA-FISH approaches to the search for molecular targets in human tumor tissues and illustrated TMPRSS2:ERG translocation by in situ prostate carcinoma (57). The MW-FISH and repeated FISH procedures will definitely become valuable tools for achieving such tasks.
forced us to feed therapy-oriented pathological information back to clinicians in daily practice, in addition to traditional, detailed morphological descriptions of tumors. Accumulation of genome-wide amplification profiles of the tumors will provide another arsenal for pathologists, especially in the era of high-throughput methodology and refinements in the genetic atlas of tumors. Finally, although only descriptive, information in ‘real’ human tumors, possibly in their incipient stage, should facilitate mechanistic or manipulative studies with particular identified genes, and more importantly, lead to the discovery of clues that lead to a cure. The MW-FISH protocol described above is a modification of the present available protocols, but since the huge amount of tumor tissue resected every day are not intentionally processed for research purpose, the MW-FISH will provide a great benefit to survey or determine genetic changes in human tumors. The situation in which pathologists find themselves today continues to be a place where ‘In pathology, you quite commonly find unusual things’ (J. Robin Warren, Nobel Prize in Physiology or Medicine, 2005).

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References


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