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In situ detection of precursor and mature microRNAs in paraffin embedded, formalin fixed tissues and cell preparations ☆

Gerard J. Nuovo *

Department of Pathology and the Comprehensive Cancer Center, Ohio State University Medical Center, 81 HLRI, 473 W 12th Avenue, Columbus, OH 43210, USA

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Abstract

The in situ detection of microRNAs (miRs) expression offers several challenges. It would be advantageous to have a method which can be used in paraffin embedded, formalin fixed tissue to be able to access the large data bank of archival material. Further, it would be helpful if one could differentiate between precursor and mature, active forms of the miR. In this review, two different methods for the in situ detection of miR in paraffin embedded, formalin fixed tissues are described. Detection of the inactive precursor miR can be accomplished by RT in situ PCR. This will allow the detection of one copy of a given pre-miR per cell. Detection of the mature form of a given miR can be accomplished with in situ hybridization with a labeled probe in which some of the nucleotides have been modified; this is referred to as a locked nucleic acid (LNA) probe. An intense signal after in situ detection with the LNA probe documents marked up-regulation of the, typically, mature miR. Further, one can easily determine the specific subcellular compartmentalization of the precursor and mature forms which may provide insight into the modulation of these important regulatory molecules and their targets.

Keywords: MicroRNA precursor; In situ hybridization; In situ PCR; LNA probes

1. Introduction

MicroRNAs (miRs) are small, noncoding sequences of around 20–23 nucleotides that regulate cell processes by being able to silence specific mRNAs via annealing to their UTR. MicroRNA activity is important in the regulation of basic cell processes such as embryogenesis, cell differentiation, and oncogenesis [1–3]. When increased miR expression is associated with oncogenesis, concomitant translation repression of cell growth suppressor molecules is commonly noted [1]. Further, when decreased expression of miRs, such as miR-15 and -16, is associated with oncogenesis, there may be a concomitant increased expression of molecules, such as bcl-2, that can facilitate the development of the malignant state [4].

It has been well established that microRNA profiles, defined typically by the up-regulation of a variety of specific microRNAs, has been associated with a wide range of cancers when compared to the corresponding normal tissue [4]. MicroRNA profiles have not only been related to cancers per se, but also to prognosis in specific malignancies. For example, increased expression of microRNA 155 and decreased expression of microRNA 7 correlates with poor prognosis in patients with lung cancer [5].

Most studies on microRNA expression have used solution phase RT PCR, primarily real time RT PCR [6,7] or microchip array technology [1]. There is relatively little data on the detection of microRNAs in cancers with in situ-based systems [8,9]. The latter reflects several technical issues including the relatively low copy number of many microRNAs, especially the precursor molecule. Importantly, the small size of the mature miR, translates into a

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* Fax: +1 614 247 4790.
E-mail address: gnuovomd@pol.net

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melting temperature (T_m) of the miR/cDNA probe hybridized complex which may be too low to allow for its detection during the various steps of the in situ hybridization process [10]. This issue can be addressed with the locked nucleic acid (LNA) modification of the nucleotide bases, which can markedly increase the T_m of these small (20 base pair) probe/target complexes. Nonetheless, even though the increased expression of several mature miRs has been associated with certain malignancies, documentation that only the malignant cells and not the adjacent normal tissue is expressing the miR of interest is often lacking. Further, information on the specific subcellular compartmentalization of most miRs is also minimal. One could use RT in situ PCR to localize the precursor of the miR in situ; a negative result would effectively rule out production of the precursor in a given cell as this method has a detection threshold of one copy per cell [10]. Standard in situ hybridization with a LNA modified probe that is complementary to the mature miR could detect both the precursor and mature form though, as will be discussed, a signal with the LNA probe typically indicates increased expression of the mature miR. This method has a detection threshold of around 20 copies per cell [10]. The purpose of this review is to provide detailed step-by-step protocols for detection of miRs in situ using both RT in situ PCR and in situ hybridization with LNA modified probes.

2. Materials and methods

2.1. Cell/tissue fixation

All tissues and cell preparations were immediately fixed in 10% buffered formalin for at least 4 h. The methodologies described in this review are best suited to cells/tissues that have been fixed for a minimum of several hours in a potent cross-linking fixative. Although in situ hybridization can be done on unfixed, frozen tissue (commonly called cryostat sections) or with tissue fixed with denaturing fixatives such as acetone or alcohol, the signal tends to be weaker and the cell morphology is noticeably worse when directly compared to material fixed with 10% buffered formalin [10]. Further, it should be stressed that fixatives that contain buffered formalin and other ingredients, such as picric acid (Bouin's solution) or heavy metals (Zenker's solution), are contraindicated with any in situ based method, as fixation with these molecules results in the rapid degradation of RNA and DNA [10–12].

2.2. General statements

Fixation with a fixative such as 10% buffered formalin will cross-link proteins to themselves as well as to DNA and RNA. The resultant adducts will reduce the availability of the target miR to the cDNA probe. To "unmask" the miR of interest, one must in a controlled fashion either denature proteins or degrade proteins and, thus, allow direct access of the probe to the miR of interest. This is most commonly done with a protease digestion step. Although cell conditioning, which involves exposing the tissue/cells to high heat prior to in situ hybridization, can be used, we prefer protease digestion as this is easier to do, requires no special equipment, and there is extensive data on the relationship of protease digestion time to the signal with both in situ hybridization and RT in situ PCR [10].

There are several "tricks of the trade" that are helpful to know when performing experiments using either in situ hybridization or RT in situ PCR. When using in situ hybridization, one can employ the same protease digestion conditions (e.g., 2 mg/ml of pepsin for 30 min at room temperature) and obtain a signal in over 90% of tissues for a given target [13]. This leads to the obvious question: what variables are important to adjust with in situ hybridization if one is either not obtaining a strong signal in a tissue known to contain high copy numbers of the target and/or if one is noting high background in a tissue known not to contain the target? The variables that can be adjusted to address these issues are: probe size, probe concentration, hybridization time, and post hybridization stringency conditions.

One tries to use probes that are long enough to allow for as many hydrogen bonds between matched base pairs as possible, which will maximize the T_m of the probe/target complex, while still allowing the probe easy access to its target in the cytoplasm or nucleus. The optimal size range for a probe and in situ hybridization is between 60 and 110 nucleotides [10,13]. If the probe is too large, the signal may be reduced due to poor entry into the cell. If it is too small, there is an increased risk that the signal will be diminished because there are not enough base pairs in the hybridized complex to favor hybridization over denaturation during the hybridization and washing steps. Further, small probe/target complexes invites background, as the T_m of the complex may be close to the T_m of the probe/nontarget complex which is the cause of background. One can have a probe synthesized directly and choose a size of between 60 and 110 nucleotides; new technology makes synthesis of these ultramers routine. Alternatively, one can use much larger probe templates and, with either random priming or nick translation, generate a large series of smaller probes that typically will fall into the 60–110 nucleotide range.

The optimal concentration for a probe is usually in the range of 100–1000 ng/ml. It has been my experience that this can best be determined by trial and error using various dilutions with tissues/cells known to be negative and positive for the target, respectively. As with each of these variables, one attempts to maximize signal relative to background.

When one compares the time of hybridization to the generation of signal and background, typically signal will begin at around 30 min of hybridization and maximize at between 2 and 3 h [10,13]. Thus, for a high copy target, such as human papillomavirus (HPV) in low grade cervical dysplasias, one may get a strong signal after 30 min of