RESEARCH ARTICLE

miR-205 in Situ Expression and Localization in Head and Neck Tumors - a Tissue Array Study

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Abstract

Background: microRNAs are small non-coding RNA that control gene expression by mRNA degradation or translational inhibition. These molecules are known to play essential roles in many biological and physiological processes. miR-205 may be differentially expressed in head and neck cancers; however, there are conflicting data and localization of expression has yet to be determined. Materials and Methods: miR-205 expression was investigated in 48 cases of inflammatory, benign and malignant tumor tissue array of the neck, oronasopharynx, larynx and salivary glands by Locked Nucleic Acid in situ hybridization (LNA-ISH) technology. Results: miR-205 expression was significantly differentially expressed across all of the inflammatory, benign and malignant tumor tissues of the neck. A significant increase in miR-205 staining intensity ($p<0.05$) was observed from inflammation to benign and malignant tumors in head and neck tissue array, suggesting that miR-205 could be a biomarker to differentiate between cancer and non-cancer tissues. Conclusions: LNA-ISH revealed that miR-205 exhibited significant differential cytoplasmic and nuclear staining among inflammation, benign and malignant tumors of head and neck. miR-205 was not only exclusively expressed in squamous epithelial malignancy. This study offers information and a basis for a comprehensive study of the role of miR-205 that may be useful as a biomarker and/or therapeutic target in head and neck tumors.

Keywords: miR-205 - head and neck cancers - FFPE - locked nucleic acid - in situ hybridization - tumor tissue array

Asian Pac J Cancer Prev, **15 (21)**, 9071-9075

Introduction

Head and neck cancers, which include cancers identified in the oral cavity, pharynx, nasal cavity, paranasal sinuses, salivary glands and larynx, is the sixth most common cancer worldwide (Jemal et al., 2011). At least half a million new cases were diagnosed annually thus demand for deeper understanding of its molecular carcinogenesis in order to hasten discovery of novel biomarkers for early detection and potential development of targeted intervention (Babu et al., 2011; Jemal et al., 2011). An escalating number of potent structural and functional genomics-based studies are starting to reveal diverse types of genetic alterations associated with these malignancies (Babu et al., 2011).

One of the major breakthroughs in genetic alteration associated with cancer development is the discovery of microRNAs (miRNAs). miRNAs are endogenous, small, non-coding RNA with approximately 18-25 nucleotides in length (Lagos-Quintana et al., 2001; Lee and Ambros, 2001). miRNAs regulate target gene expression by mRNA degradation or translational inhibition, and are known to play important roles in many biological and physiological processes including cell division, development and apoptosis (Ouyang et al., 2012). The latest version of miRNA database, miRBase20 reported 24,521 microRNA loci from 206 species, producing 30,424 mature microRNA products (Kozomara and Griffiths-Jones, 2014). There are 2,578 miRNAs discovered in human to date and more than a hundred have been implicated in various human diseases including cancer (Krutovskikh and Herceg, 2010; Kozomara and Griffiths-Jones, 2014). Differential expressions of cancer-associated miRNAs have been reported in various cancer including carcinomas of the head and neck (Krutovskikh and Herceg, 2010). Oncogenic and tumor suppressor miRNAs have been described (Spizzo et al., 2009; Ventura and Jacks, 2009) and over-expression of oncogenic miRNAs and down regulation of tumor suppressor miRNAs have been shown to make vital contributions to tumor formation, invasion as well as metastasis (Sassen et al., 2008; Baranwal and Alahari, 2010).

Majority of miRNA expression studies utilized solution phase RT-PCR, mainly qRT-PCR (Benes and Castoldi, 2010), microarray and next generation sequencing technology (Git et al., 2010). The major advantage of an in situ hybridization (ISH) system over other miRNA detection systems is that the tissue is not destroyed before the molecular analyses (Nuovo et al., 2009). In addition, in situ analysis enables the identification of specific cell

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type(s) that contain the miRNA of interest as well as the determination of the sub-cellular compartment that contains the particular miRNA (Nuovo et al., 2009). Hence, miRNA in situ analysis may possibly play an important role in the clinical laboratories for cancer diagnosis and therapeutics (Nuovo et al., 2009).

There is little data on miRNAs detection in cancers using in situ-based systems (Politz et al., 2006; Nuovo, 2008). In head and neck cancers, there are only a few studies involved the use of miRNAs ISH analysis (Harris et al., 2012; Tu et al., 2012; Liu et al., 2014; Lu et al., 2014) and were performed on specific head and neck cancer subtype. Apart from being more common in Asian region thus getting less attention from other continents (Mehanna et al., 2010), this reflects a number of issues, including the small size of the mature miRNA that results in a low melting temperature (Tm) of the miRNA-DNA probe-hybridized complex which is too low to permit for its in situ detection (Nuovo et al., 2009). However, the Tm issue can now be overcome using LNA modification of the nucleotide bases in the miRNA detection probe. The LNA modification allows for an increase in Tm of approximately 20-30°C if six nucleotides in the probes are LNA-modified (Obernosterer et al., 2007; Nuovo, 2008). Locked Nucleic Acid in situ hybridization (LNA-ISH) is a novel technology for the miRNA detection in FFPE human cancer tissues with superior sensitivity and exceptional specificity (Zhang et al., 2011).

miR-205 expression is dysregulated in many human cancers, influenced various cancer cell properties in cell lines and also regulated several major cancer pathways (Xie et al., 2012; Lei et al., 2013; Su et al., 2013; Wang et al., 2013). However, pattern of miR-205 expression in head and neck cancers demonstrated conflicting results. In addition, there is no information available on its localization of expression by ISH. miR-205 was shown to have high expression level in head and neck squamous cell carcinoma cells lines (Tran et al., 2007). This was further supported by a more recent finding whereby miR-205 showed highest expression in both malignant and benign squamous epithelia while was being least expressed in cell lines and tissues other than squamous type (Kimura et al., 2010). On the contrary, Childs and colleagues proved low expression level of miR-205 in tumor tissues compared with the normal adjacent tissues (Childs et al., 2009). Thus, to better understand the involvement of miR-205 in head and neck carcinogenesis, this present study aims to visualize miR-205 expression patterns and localization in various cases of inflammatory, benign and malignant tumor tissues of head and neck using LNA-ISH on the formalin-fixed, paraffin embedded (FFPE) tissue sections.

Materials and Methods

Head and neck tissue array

Human head and neck tissue array was purchased from BioChain Institute (Hayward, CA, USA; Catalog No.: Z7020051, Lot No.: B110059) and consisted of 48 duplicate cases of inflammatory, benign and malignant tumor tissues of the neck, oranasopharynx, larynx and salivary glands. This tissue microarray contained 15 cases of squamous cell carcinoma of the head and neck; eight pleiomorphic adenoma cases; five cases of adenoid cystic carcinoma; three cases for each inflammatory and polyps; two cases for each of hemangioma, neurofibroma, schwannoma and metastatic adenocarcinoma cases and one cases for each of mucoepidermoid carcinoma, rhabdomyosarcoma, chondrosarcoma, B-cell lymphoma, diffuse large B-cell lymphoma and metastatic squamous cell carcinoma.

Hematoxylin and eosin staining

Hematoxylin and Eosin (H and E) staining was performed to confirm the diagnosis, to identify representative tumor regions and for comparison with ISH results.

LNA-modified oligonucleotide probes

Double DIG-labeled LNA probe for miR-205 and scramble miRNA LNA probe (as a negative control) as well as microRNA ISH buffer were purchased from Exiqon (Vedbaek, Denmark). To prepare 50 μL of 2 pmol/ μL LNA probes for each slide, 4 μL of 25 μM LNA Detection probe was transferred into a 0.2 mL non-stick RNase-free tube and was placed at 90°C for 4 min. Forty six microlitres of 1x microRNA ISH buffer (Exiqon, Vedbaek, Denmark) was immediately added into each of the tubes with the different LNA probes. Precaution step was taken not to re-freeze the diluted LNA-probes.

miRNA LNA ISH

Protocols for miRNA LNA-ISH were adapted from Nuovo (Nuovo, 2008) with several modifications. Firstly, four-micrometer-thin sections of FFPE tissues adhered to glass slides were deparaffinized by dipping the slides for 5 min in fresh xylene. Dehydration step followed by dipping the slides for 5 min in absolute ethanol and then air-dried. The next step is protease digestion of the tissue sections. The slides were placed on a flat surface and a hydrophobic barrier around tissue sections was made using a Super PAP pen (MP Biomedicals, Orangeburg, NY). Approximately 500 μL/slide of 15 μg/ml Proteinase K (Bio Basic Inc., Ontario, Canada) was applied on the tissue sections and the slides were incubated for 10 min at 37°C. Then the slides were placed in 1x phosphate buffer saline (PBS) solution for 30 sec to stop the Proteinase K treatment, followed by dehydration of the slides in absolute ethanol for 30 sec. The slides were air-dried for approximately 15 min. The separate tissue sections are then covered with the probe cocktail which, in turn, are covered with sterile polypropylene coverslips cut to the size of the slides. Precaution was taken to avoid bubble formation. The tissue sections were incubated with the probe at 60°C for 5 min followed by hybridization for 17 h at 37°C.

On the next day, the coverslips were carefully removed and the slides were washed in 0.2% SSC and 2% bovine serum albumin (BSA) (Bio Basic Inc., Ontario, Canada) solution at 4°C for 10 min. The slides were incubated for 30 min at 37°C in antidigoxigenin-alkaline phosphatase conjugate diluted to 1:200 using 10x blocking solution and 1x maleic acid buffer (all from Roche, Mannheim, Germany). Then the slides were washed in 500 μL

1x detection buffer (Roche, Mannheim, Germany) at room temperature for 1 min. Cocktail for chromogen development contained 800 μL of 1x detection buffer (Roche, Mannheim, Germany) 5 μL of 4-Nitro-blue tetrazolium (NBT; Fermentas, Lithuania) and 5 μL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP-T; Fermentas, Lithuania) for each slide. The slides were incubated in NBT/BCIP-T chromogen at 37°C for 10 min in the dark. Color development was terminated by dipping the slides in RNAse-free water. Nuclear fast red (Bio Basic Inc., Ontario, Canada) served as a counterstain to allow for better contrast with the blue signals generated by probe-target complex. The slides were placed in nuclear fast red solution for 1 min, in RNAse-free water for 30 sec, in absolute ethanol for 2 min and lastly in fresh xylene for 2 min. The slides were then air-dried, mounted with Mounting Medium (Pierce, Rockford, Ill) and covered with glass coverslips.

Image acquisition and analysis

For image analysis the Image-Pro Plus integrated microscope and software module (Media Cybernetics, Silver Spring, USA), comprising of a Nikon Eclipse 80i microscope (Nikon, Melville, NY, USA) equipped with a Q-Imaging camera (Q-Imaging, Burnaby, BC, Canada) were used. For miR-205 analysis, the stained structures were differentiated: blue staining is corresponding to the hybridization signal, red staining is corresponding to the red nuclear stain and purple staining is corresponding to blue LNA-ISH signal overlaying red nuclear stain. Slides were scored as - negative, + weak staining, ++ moderate staining and +++ strong staining by a pathologist. Adobe Photoshop (version 5.0; Adobe Systems Inc., San Jose, CA) is used only to correct brightness, contrast, color balance and to remove particulates on the images (Darnell et al., 2006). Significant difference of miR-205 signals among the cases was calculated using Student T-test (SPSS version 16, Chicago, Illinois, USA).

Results

Expression patterns of miR-205 in intact head and neck tissue array

Staining of miR-205 in the cytoplasm was observed in majority of the cases with different intensities. miR-205 also demonstrated nuclear staining in some cases. Since mature miRNAs are present in the cytoplasm (Macfarlane and Murphy, 2010), therefore the probe most likely detected the mature miRNAs signals. These results showed that LNA-ISH method could detect miR-205 in a semi-quantitative manner. Table 1 showed the average scoring of miR-205 signals in head and neck tissues.

There was no hybridization signal detected in inflammation cases. Weak to moderate signals were observed in polyps, hemangioma, neurofibroma and schwannoma. miR-205 signal was weak in pleomorphic adenoma cases whereby only 5 out of 8 cases (62.5%) showed weak staining while the rest were negative for miR-205. In SCC FFPE tissues, a total of 46% (6/13) cases showed strong miR-205 signals, 38.5% (5/13) showed moderate signals and 15.4% (2/13) demonstrated weak signals. Moderate miR-205 signals were observed in mucoepidermoid carcinoma and metastatic SCC while there were weak to moderate signals in adenoid cystic carcinoma cases. There were strong miR-205 signals in rhabdomyosarcoma and chondrosarcoma cases. Weak signals were noted in B cell lymphoma and diffuse large B cell lymphoma. There was also strong miR-205 signal in metastatic adenocarcinoma.

Significant difference of miR-205 signals among the cases obtained using Student T-test was illustrated in Table 2. A marked increase in miR-205 staining intensity was observed from inflammation to benign and malignant cases in head and neck FFPE tissues, suggesting that miR-205 could be a biomarker to differentiate between cancer and non-cancer tissues. Representative microscopic images of expression of miR-205 in head and neck tissue array by LNA-ISH were shown in Figure 1 and Figure 2.

Localization of miR-205 in head and neck tissue array

Using LNA-ISH, the type of cell(s) and cell compartment(s) that expressed miR-205 was determined. Cytoplasmic miR-205 expression was localized to the nest of SCC cells as well as in individual SCC cells in the stroma. Fibroblasts, keratin cells and red blood cells did not exhibit miR-205 signal. In metastatic SCC, cytoplasmic staining of miR-205 was observed but the basal lining was negative for miR-205. Rhabdoid cells and neoplastic cells in chondrosarcoma also showed positive miR-205 cytoplasmic staining while wavy nuclei in neurofibroma was positive for miR-205 staining. Mucoepidermoid carcinoma showed positive miR-205 signal in cytoplasm of cancerous cells but negative miR-205 signal was observe in mucous and intermediate cells. Adenocarcinoma cells also exhibited cytoplasmic miR-205 staining. In addition, positive nuclear miR-205 staining was observed in endothelial cells lining the blood vessel. Representative microscopic images of miR-205 localizations in head and neck tissue array by LNA-ISH were shown in Figure 3.

Discussion

This is the first study that combines a panel of head and neck diseases and in situ hybridization to evaluate the expression and localization of miR-205 expression. Generally, miR-205 was demonstrated to be most frequently highly expressed in the cytoplasm of malignant cells of the head and neck cancers and not only expressed in SCC cells as proposed by other study (Fletcher et al., 2008).

Increasing evidences have illustrated the complex roles of miR-205 in tumor initiation and progression (Orang et al., 2014). miR-205 is a tumor suppressor by inhibiting proliferation and invasion, but also as an oncogene by aiding in tumor initiation and proliferation, depending on the particular context of tumor. miR-205 is known as potentially essential miRNA in head and neck tumorigenesis (Tran et al., 2010). Jiang and colleagues observed that miR-205 was shown to be over-expressed only in head and neck cancer cell lines when compared to other cancer cell lines from the lung, breast, colorectal,

prostate, and pancreas (Jiang et al., 2005). Another study concluded that miR-205 may be solely over-expressed in head and neck tumors (Tran et al., 2007). In addition, miR-205 expression was higher in frozen nasopharyngeal cancer tissue compared to adjacent normal nasopharynx tissue (Chen et al., 2009). Our findings provide other perspectives to the previous studies whereby miR-205 was shown to be absent in non-cancerous (inflammation) tissues while its highest expressions were observed in malignant head and neck tumors. All these findings pointed to oncomiR property of miR-205 in head and neck cancers.

miR-205 has been known to be highly expressed in HNSCC cell lines relative to other tumor types (Tran et al., 2010) while Fletcher and colleagues demonstrated that its expression is highly specific for squamous epithelium (Fletcher et al., 2008). On the contrary, Childs and colleagues proved low level of miR-205 expression in HNSCC (Childs et al., 2009). These diverse expression patterns in certain types of cancer might reflect the existence of tumor type-specific mechanisms that are mediated by miRNAs. However, our study revealed that miR-205 was not exclusively expressed in squamous epithelial malignancy. Rhabdomyosarcoma, chondrosarcoma, and metastatic adenocarcinoma in head and neck region also showed high cytoplasmic miR-205 expression. Nevertheless, this may be due to small number of the cases analyzed in this study thus follow up investigation is necessary to validate this finding. These data are novel in a sense that miR-205 was also observed in non-squamous carcinoma of head and neck.

Another interesting finding from this study is the ability of miR-205 to differentiate intermediate cells from squamoid and mucinous component in mucoepidermoid carcinoma (MEC). Mucoepidermoid carcinoma is composed of mucous secreting cells, epidermoid type (squamoid) cells and intermediate cells. The mucous cells have abundant pale, foamy cytoplasm that stain positively for mucin stains while the epidermoid cells possess squamoid features, polygonal shape, intercellular bridges and rarely keratinization. A population of cells that is more crucial in identifying MEC is a group of highly prolific, basaloid cells referred to as intermediate cells. These cells are identified as being larger than basal cells but smaller than the squamous cells, and are alleged to be the progenitor of epidermoid and mucous cells (Wolfish et al., 2012). Current tool in differentiating MECs from other salivary gland carcinomas that they may strongly mimic is by recognizing intermediate cells via immunohistochemistry (Schwarzet al., 2011). In situ analysis revealed strong cytoplasmic miR-205 staining in squamoid cells, but none in adjacent intermediate cells. Our study suggests that miR-205 could also be a biomarker in differentiating intermediate cells from epidermoid component in MEC.

Several studies had proven the involvement of many miRNAs in head and neck tumorigenesis (Avissar et al., 2009; Liu et al., 2009; Nohata et al., 2011a; Nohata et al., 2011b). However, only a few had discussed the miR-205 involvement in head and neck cancers (Tran et al., 2007; Fletcher et al., 2008; Childs et al., 2009; Kimura et al., 2010) and the conclusion remains uncertain. This study showed a strong involvement of miR-205 in head and neck cancers. Even though the mechanisms underlying miR-205 localization in malignant cells remained to be determined, targeting this miRNA could be a potential strategy in managing head and neck cancers.

In conclusion, using the LNA-ISH for miRNA detection, miR-205 was detectable in majority of head and neck diseases with different intensities. miR-205 demonstrated significant differential cytoplasmic and nuclear staining among inflammation, benign and malignant head and neck cases. These results offer information and provide basis for an in-depth study of the role of miR-205 that may be useful as a biomarker and/or therapeutic target in head and neck tumors.

Acknowledgements

The authors thank Associate Professor Shiran Mohd Sidik from Perdana University Graduate School of Medicine for the guidance in the scoring of ISH slides.

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