(Extended Abstracts)

The role of methylation in the regulation of MicoRNA in bladder cancer

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Abstract:

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate gene expression at the post-translational level. Recent studies have shown that miRNAs can play a key role at the early stages of cancer development and progression. Furthermore, studies have suggested that epigenetic silencing of miRNAs with by DNA hypermethylation may contribute to the development of human bladder cancer. To identify such miRNAs in bladder cancer, expression and methylation patterns will be analyzed in bladder cancer cell lines treated with the commonly used demethylating agent. The generated results will reveal the potential role of miRNAs in the treatment and prediction of bladder cancer progression.

Key words: Epigenetic - Methylation – Microarray- MicroRNA – Bladder cancer.

Introduction:

Title: The role of methylation in the regulation of MicroRNA in bladder cancer

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Bladder cancer is a commonly occurring cancer, ranking as fourth in the UK and as ninth worldwide in terms of overall cancer incidence. In 2006, it was estimated that more than 356,600 individuals per year were diagnosed with bladder cancer worldwide. Industrially developed populations such as those in Western Europe and North America are more prone to bladder cancer and it also found frequently in countries where

schistosomiasis is endemic such as Africa and the Middle East. In addition, males are more susceptible to bladder cancer than females, with a worldwide ratio of 10:3 male-female. There are a number of risk factors associated with bladder cancer including tobacco consumption, occupational exposure, medical conditions and nutritional habits (Cancer research UK, 2006).

The bladder consists of four layers: epithelial, lamina propria, muscle layer and fat layer. The depth of tumour penetration in to the bladder wall is an important prognostic factor and is identified by t-staging of bladder cancers (Ta- papillary non-invasive papillary carcinoma; Tcis - Carcinoma in situ (flat non-invasive carcinoma); T1 - tumour localised on urothelial lining; T2 - tumours grown into the muscle layer; T3 - tumour penetrates into the fatty layer surrounded the bladder and T4 tumours have spread to beyond the fatty tissue and invaded other tissues such as prostate, uterus, vagina and pelvic wall). Approximately 90% of bladder cancers are transitional urothelial cell carcinomas, whereas 10% are squamous cell carcinomas or adenocarcinomas. The majority of transitional urothelial carcinomas are superficial diseases with 70% requiring local resection. The remaining 30% of urothelial carcinomas are muscle invasive at early clinical presentation (Lopez-Beltran, 2008).

Chromosomal mapping studies of bladder cancer have suggested that a number of tumour suppressor genes are located on chro19q (Simoneau *et al,* 1996). Moreover, defects or deletions in this key regulatory region may play critical role in developing bladder cancer. These genetic alterations could reflect differences between superficial and invasive bladder cancers such as high frequency of chromosome 9 loss of heterozygosity (LOH) and low frequency of the p53 tumour suppressor gene which have been observed in papillary tumours. In contrast, high frequency of p53 mutation and defect in chromosome 9 leads to the development of invasive tumours (Gonzalgo *et al*, 2007).

High throughout genomic studies have revealed that >90% of the human genome is transcribed, although the majority of these transcripts do not encode proteins and are ”noncoding transcripts”. These discoveries revealed a new class of short RNAs known as ’MicroRNAs’. MicroRNAs (miRNAs) are class of endogenously produced, small (18~25 nucleotides), noncoding RNAs. They are highly conserved and play essential role in variety of physiological processes in cell including differentiation, proliferation, apoptosis,

immunity and metabolism. MiRNA usually binds to the 3' untranslated region (3' UTR) of their targeted messengerRNA (mRNA) and generally down regulates gene expression, either by inducing mRNA degradation or by inhibiting translation (He and Hannon, 2004).

Recent studies have identified that miRNA expression differs between normal and cancer cells, suggesting that miRNAs can act as tumour suppressors or as Oncomirs (Esquela-Kerscher and Slack, 2006). Calin *et al*, first identified a miRNA with tumour suppressor properties in a patient with B-cell chronic lymphocytic leukaemia (B-CLL). In this study, it was found that mir-15a and mir-16-1 were down regulated in B-CLL patients. Moreover, both mir-15a and mir-16-1 negatively regulated the 'antiapoptotic protein' BCL2 at the post-translational level (Calin *et al,* 2002; Cimmino *et al.,* 2005). In contrast, other studies have shown that over expression of mir-17-92 causes the development of malignant lymphoma in mice (He *et al,* 2005), and mir-21 has been shown to be up-regulated in glioblastoma and breast cancer (Chan *et al,* 2005; lorio *et al,* 2005).

There are few reported studies investigating the role of miRNAs in bladder cancer. A Study carried out in 25 urothelial carcinomas and 2 normal mucosal tissues (Gottardo *et al*, 2007), identified 10 up- regulated miRNAs in urothelial carcinoma compared to normal mucosa. Moreover, analysing changes in miRNA expression between superficial and invasive bladder carcinomas identified 9 differentially expressed miRNAs in invasive and non-invasive carcinomas (Neely *et al*, 2008). Furthermore, the expression ratio of mir-21:mir-205 was 10-fold higher in invasive tumours than non-invasive, suggesting that miRNAs could be an important prognostic marker to distinguish different stages in bladder cancer. Interestingly, a recent study has also shown mir-143 becomes down-regulated in bladder tumour cells in comparison to normal cells. When mir-143 was transfected into the bladder cancer cell lines, cell proliferation was significantly reduced, demonstrating that mir-143 may act as a tumour suppressor (Lin *et al,* 2009).

DNA methylation is part of epigenetic regulation in human genome. However, dramatic changes in DNA methylation at the global or regional level are among frequently occurring events in human cancer. DNA methylation is a reversible enzymatic reaction which is catalysed by DNA methyltransferases (DNMTs) in which the 5'-position of cytosine becomes methylated. In mammals, DNA methylation causes long-term silencing of genes by histone modifications and alterations in chromatin structure. It most frequently occurs at

GC rich regions known as CpG islands which are usually found at the 5' end and can include the promoter, exon1 and the untranslated region. In normal cells, CpG islands tend to be hypomethylated whereas, CpG poor regions are usually methylated. In contrast, during early stages of cancer development CpG poor regions undergo hypomethylation while CpG islands become hypermethylated (Miranda and Jones, 2007).

Epigenetic gene silencing has previously been shown to be an important factor in the progression of bladder cancer, with tumours displaying a high frequency of gene inactivation appearing more aggressive and refractory to treatment than other cancers. The promoter of number of tumour genes are known to be hypermethylated in bladder cancer, including E-cadherin, p16 and ras-associated factor 1A (RASSF1A) (David *et al,* 2007). Recent studies have established that tumour suppressor genes silenced due to hypermethylation could be reactivated using epigenetic drugs such as 5-azacytidine (Vidaza) and 5-Aza-2-deoxycytidine (Dacogen) (Datta *et al,* 2008; Lujambio *et al,* 2008).

Hypothesis:

The complexity of microRNA epigenetic regulation has opened a new era in our understanding of the molecular development and regulation of cancer. In the present study we hypothesise that hypermethylation of miRNAs may lead to bladder cancer progression, whilst using demethylating agents may reactivate silenced miRNA molecules and inhibit cancer progression. A study by Datta *et al*. reported that mir-1-1 was silenced due to hypermethylation in the human hepatocellular carcinoma (HCC) cell line. However, after treating these cell lines with 5-azacytidine and/or trichostatin A (epigenetic drugs), they identified reactivation of mir-1-1 which led to inhibition of tumour cell growth and down regulation of oncogenic targets.

Materials and Methods:

To investigate this hypothesis in bladder cancer, the miRNA expression profile in 3 human urothelial carcinoma cell lines representing three different stages of bladder cancer (EJ, RT4 and RT112) will be analysed. These cell lines have been treated with 5-azacytidine (DNA demethylating agent). MicoRNA and total RNA were extracted using the mirVANA extraction kit (Applied Biosystems).

The isolated miRNA from treated and untreated cancer cell lines will be hybridized to the Agilent miRNA microarray chip (Agilent microarray Rel12.0 Sanger miRBase, v.12.0) to investigate differentially

expressed miRNAs, furthermore, bioinformatics tools such as GeneSpring (Agilent) will be used for validation and identifying miRNA targets.

Commercially available kits (Qiagen) will be used to isolate DNA from urothelial carcinoma cell lines and methylation changes will be detected by bisulphite conversion. Regions surrounding differentially expressed candidate miRNA obtained from microarray analysis will undergo bisulfite genomic sequencing to identify the presence of neighbouring methylated CpG islands. (Toyota *et al.,*2008).

Summary:

Bladder cancer is debated to be the most expensive human cancer to mange. Unfortunately, currently there are no any sufficient and robust clinical procedures for cancer surveillance with low risk of disease. The majority of patients with non-life threatening tumours are required to undergo regular cystocopies which can cause morbidity and discomfort. The miRNA profiling has emerged not only as a potential diagnostic and prognostic indicator in cancer but also as therapeutic target for cancer treatment. Antisense oligonucleotides (ASOs) could be used to design complementary miRNAs which could inhibit putative oncomirs. In addition, epigenetic drugs could be used to reactivate silenced miRNAs which have become hypermethylated.

The current understanding of the molecular mechanisms of miRNA in cancer regulation is in its infancy, particularly in bladder cancer. As few very studies have been published on miRNA and bladder cancer, our study will contribute to understanding the role of epigenetic regulation of miRNA in bladder cancer.

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