COMPARATIVE RE SITES STUDY OF HUMAN TUMOR SUSCEPTIBILITY GENE 101 (*TSG101*) FOUND AMONG DIFFERENT HUMAN TISSUE TYPES

ANUSYAH A/P RATHAKRISHNAN

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Faculty of Resource Science and Technology
UNIVERSITY MALAYSIA SARAWAK
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LISTING OF ABBREVIATIONS

^{o}C	Degree Celsius
µl	microlitre
µg	microgram
bp	Base pairs
cDNA	Complementary Deoxyribonucleic Acid
ddH_2O / sdH_2O	Double distilled Water / Sterile Distilled Water
dNTP	Deoxyribonucleotide triphosphate
Dept	Department
Kb	Kilo base pairs
KCl	Kalium Chloride
Lab	Laboratory
MDM2	Mouse Double Minute 2
$MgCl_2$	Magnesium Chloride
ml	millilitre
M-MLV	Moloney-Murine Leukemia Virus
NPC	Nasopharyngeal cancer
Pr1	Primer set 1
Pr2	Primer set 2
Pr3	Primer set 3
RE	Restriction enzymes
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SGH	Sarawak General Hospital
T_a	Annealing temperature
TAE	Tris-acetate-EDTA
TSG101	Tumor Susceptibility Gene 101
UNIMAS	Universiti Malaysia Sarawak
UV	Ultra Violet
V	volts

COMPARATIVE RE SITES STUDY OF HUMAN TUMOR SUSCEPTIBILITY GENE 101 (*TSG101*) FOUND AMONG DIFFERENT HUMAN TISSUE TYPES

Anusyah a/p Rathakrishnan

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

There are many genes that encode proteins for cell proliferation, differentiation, and development. Mutations in these genes can affect its function. Accumulation of genetic alteration through mutations or epigenetic event plays an important role in cancer progression. One such gene presumed to be linked to cancer is the Tumor Susceptibility Gene 101 (*TSG101*). However, many research studies failed to detect genomic alteration in this gene yet these studies have shown that the *TSG101* undergoes alternative and aberrant splicing. Therefore, this study was proposed to detect whether the nucleotide variations and/or mutations of the *TSG101* diverge in different human tissue types via restriction site characterization. Besides that, it is also to observe whether any of the nucleotide variations dominate certain cancer types. Briefly, total RNA from nasopharyngeal carcinoma, oral carcinoma and bladder carcinoma was transcribed into cDNA prior proceeding to polymerase chain reaction (PCR) using several sets specific of *TSG101* primer. Subsequently, the PCR product was purified and subjected to restriction enzyme digestion. Agarose gel electrophoreses analysis of *TSG101* partial sequence indicated that there is no mutation found at the specific restriction site in all tissues samples studied. Hence, mutational alteration might not be the main event in all the cancer studied. Nevertheless, sequencing of the *TSG101* full length will provide more accurate and reliable results. Epigenetic studies of *TSG101* can be considered to further elucidate the genetic alteration of this gene that leads to cancer.

Keywords: *TSG101*, restriction enzyme digestion, mutation.

ABSTRAK

Terdapat banyak gen yang mengekod untuk protein bagi fungsi proliferasi, pembezaan dan perkembangan sel. Mutasi yang berlaku atas gen-gen ini boleh mempengaruhi fungsi mereka. Perubahan genetik yang terkumpul dari proses mutasi atau kejadian epigenetik memainkan peranan penting dalam perkembangan kanser. Salah satu gen yang dihubungkaitkan dengan kanser ialah Tumor Susceptibility Gene 101 (*TSG101*). Kebanyakan penyelidikan yang dijalankan gagal menemui sebarang perubahan genomik dalam gen ini namun ada penyelidikan yang menunjukkan bahawa *TSG101* menjalani percantuman alternatif dan juga percantuman abnormal. Sehubungan itu, kajian ini telah diusulkan untuk mengesan sama ada *TSG101* mempunyai variasi nukleotida dan/atau mutasi berbeza di dalam tisu manusia yang berlainan. Selain itu, kajian ini juga bertujuan melihat sama ada variasi-variasi ini mendominasi tisu kanser yang tertentu. Secara ringkasnya, RNA penuh dari carcinoma nasopharygeal, oral, bladder dan colon telah ditranskripkan ke cDNA yang kemudiannya digunakan dalam proses rantaian polymerase (PCR) dengan beberapa set primer *TSG101* yang spesifik. Produk PCR kemudiannya ditulenkan dan dilalukan percernaan enzim restriksi. Proses elektroforesis gel agarose bagi jujukan partial *TSG101* telah menunjukkan bahawa tiada sebarang mutasi berlaku di lokasi spesifik kepada enzim restriksi untuk semua jenis tisu manusia yang dikaji. Oleh yang demikian, boleh dikatakan yang perubahan jenis mutasi dalam *TSG101* bukan kejadian utama dalam kanser-kanser yang dikaji. Namun demikian, proses penjujukan bagi koding *TSG101* akan memberi keputusan yang lebih jitu dan sahih. Selain itu, kajian epigenetik *TSG101* boleh dipertimbangkan untuk penjelasan perubahan genetik yang berlaku pada gen ini yang menyebabkan kanser.

Kata Kunci: *TSG101*, percernaan enzim restriksi, mutasi.

CHAPTER 1

1.0 INTRODUCTION

Cancer is the more virulent form of neoplasia, a disease process characterized by uncontrolled cellular proliferation leading to a mass or tumour and it has to be malignant in order to be termed cancer (Nussbaum *et al.,* 2004).

Cancer is presumed to occur through a multi-step process involving sequential cycles of mutation and selection (Calabrese *et al.,* 2005). Different types of mutations are responsible for causing cancer which include mutations such as activating gain of function mutations of one allele of a proto oncogene, loss of function of both alleles or dominant negative mutations of one allele of tumour suppressor gene, and chromosomal translocation that will alter the expression of genes or create chimeric genes encoding proteins that have gained more than one functional property (Nussbaum *et al.,* 2004). These genetic abnormalities affect a number of identified pathways that are involved in carcinogenesis (Lüchtenborg *et al.,* 2005).

There are many genes that encode proteins of cell proliferation, differentiation, and development. If these genes undergo mutations, their functions will be affected (Sherr, 2004). Once initiated, a cancer progresses by accumulating other genetic damage through more mutations or epigenetic silencing of the genes that encode the cellular machinery that repair damaged DNA and maintains cytogenetic normality (Nussbaum *et al.,* 2004).

One such gene presumed to be linked to cancer is the Tumor Susceptibility Gene 101 (*TSG101*). The *TSG101* was initially thought to be a tumour suppressor gene. However, many research studies that were conducted failed to detect genomic alteration yet these studies have shown that this gene undergoes alternative and aberrant splicing. Some studies have shown the gene to be overexpressed in certain carcinomas.

Therefore, this proposed study is to detect whether the nucleotide variations and/or mutations of the *TSG101* diverge in different human tissue types. Besides that, it is also to observe whether any of the nucleotide variations dominate certain cancer types.

In this study, a restriction sites comparison of the *TSG101* among different tumour tissues will be made using restriction enzyme analysis, which is suitable for direct detection of mutations (Cotton, 1997). In most studies done, the researchers have opted for DNA sequencing to screen for nucleotide variation. However, DNA sequencing is a costly method, therefore in this study a cheaper but highly reproducible method (Strachan and Read, 1999) which is restriction enzyme analysis will be used. According to Cotton (1997), a mutation can be detected by restriction analysis by the alteration in the range of fragments seen on gel electrophoresis.

Restriction enzyme analyses are performed using Type II restriction endonucleases which are isolated from a wide diversity of bacterial genera (Hill, n.d.). Basically, restriction endonucleases are defined as double stranded DNases that recognize specific DNA sequences and make double stranded cleavage at defined point within or close to that sequence (Roberts and Halford, 1993; Fuchs and Blakesley, 1989).

1.1 OBJECTIVES

The main objective of this study is to compare the specific bands produced from the restriction enzyme digestion of the amplified gene segment for all available normal and tumour specimens to screen for nucleotide variation in *TSG101*.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Tumor Susceptibility Gene 101 (*TSG101*)

The human Tumor Susceptibility Gene 101 (*TSG101*) has been mapped to chromosome 11p15.1-p15.2 (Li *et al.,* 1997 cited in Wagner *et al.,* 1998). This site has shown loss of heterozygosity in many different types of tumours (Wagner *et al.,* 1998). The *TSG101* gene has 10 exons which is about 46.63 Kb (Genatlas, 2006). *TSG101* encodes a 391 amino acids protein (Genatlas, 2006). This protein is usually localized in the cytoplasm (Zhong *et al.,* 1997 cited in Wagner *et al.,* 1998). However, it can also be detected in the nucleus depending on the stage of the cell cycle and it can also co-localize with the mitotic spindle apparatus during cell division (Xie *et al.,* 1998 cited in Wagner *et al.,* 1998).

2.2 Structure and Function of Tumor Susceptibility Gene 101 (*TSG101*)

The *TSG101* has a few highly conserved regions, which are:

i. A C-terminal coiled coil domain which is said to interact with stathmin which is cell growth regulating protein. (Maucuer *et al.,* 1995 cited in Wagner *et al.,* 1998; Krempler *et al.,* 2002). This region is also said to be involved in a potential co-repressor activity (Krempler *et al.,* 2002).

ii. A proline rich region known to exist in activation domains of transcriptional factors (Wagner *et al.,* 1998).

iii. The N-terminal region which is similar to the catalytic domain of ubiquitin conjugating enzymes. This suggests the potential of *TSG101* as a regulator in ubiquitin mediated protein degradation. (Koonin and Abagyan, 1997; Ponting *et al.*, 1997 both cited in Wagner *et al.,* 1998). The ubiquitin conjugating enzymes are dominant negative regulators of the cell cycle control (Krempler *et al.,* 2002). Besides that, Krempler and his co-workers (2002) also stated that this domain is said to interact with the cell cycle regulator *MDM*2.

According to Wagner *et al*. (1998), due to the highly conserved motifs, *TSG101* is believed to have different functions at specific stages of the cell cycle. The authors also mentioned that due to *TSG101* potential as a regulator of protein regulation, it could influence the half life of other tumour suppressor genes and regulatory protein of the cell cycle. In one such case, Li *et al*. (2001) have pointed out that the *TSG101* participates with *MDM*2 in a separate feedback control loop that affects *MDM*2 stability and therefore affecting *p53*/*MDM*2 feedback regulation. Li and co-workers (2001) showed that *TSG101* is an inhibitor of ubiquitination. They had also found that *TSG101* can bind to both *p53* and *MDM*2. The *MDM*2/*p53* circuitry when deregulated will result in tumorigenesis (Liu *et al.,* 2002). This ability of *TSG101* to interact with *MDM*2 and/or *p53* has functional consequences on the steady-state levels of *MDM*2 and *p53* (Li *et al.,* 2001).

Xie *et al*. (1998) observed that the inactivation of *TSG101* was associated with abnormal microtubule organizing centres and nuclear anomalies; hence they suggested that the absence of the *TSG101* protein or the lack of individual *TSG101* domains may lead to chromosomal instability and consequently a progression of tumorigenesis (cited in Wagner *et al.,* 1998). However, according to Li *et al*. (2001) overexpression of *TSG101* mRNA in either the sense

or antisense direction can lead to neoplastic transformation, whereas the total absence of *TSG101* could result in *p53* accumulation, cell growth arrest, and early embryonic death. This was due to the complicated effects of *TSG101* on growth regulatory proteins and pathways particularly *MDM*2 and *p53* (Li *et al.,* 2001).

2.3 Bladder Cancer

The bladder is a hollow organ with flexible muscular walls located in the lower abdomen (Calvagna, 2007). It is used to store urine (Calvagna, 2007). According to second report of the National Cancer Registry in year 2003, the bladder cancer incidence per 100 000 populations in the Peninsular is 2.5 for both sexes (Lim and Halimah, 2004). It is also stated in the report that, the bladder cancer highly occurs in Malay males (52.6%) and both Malay and Chinese females (47.1%).

The cancers of bladder are named after the cell type that forms the cancer (Calvagna, 2007). There are three main cell types hence three different cancers of the bladder. First, is the transitional cell carcinoma or also known as urothelial carcinoma which are from the transitional cells forming the innermost lining of the bladder wall (eMedicineHealth, 2005) which accounts for 90% of bladder cancers (Calvagna, 2007). Second is the squamous cell carcinoma which forms from the thin, flat cells because of some form of inflammation or irritation that has taken place for many months or years (eMedicineHealth, 2005). Finally, the third type is the adenocarcinoma which is formed from the cells that make up glands (eMedicineHealth, 2005). Among the risk factors for bladder are presumed to be caused by

tobacco smoking, amount of liquid intake, occupational exposures and infection by *Schistosoma haematobium* (Tyczynski and Parkin, 2003).

2.4 Colorectal cancer

According to American Cancer Society (2005), colorectal cancer is the cancer which develops in colon or the rectum. The process of forming cancer usually begins with the non-cancerous polyps which eventually becomes cancer. Adenomatous polyps are more likely to become cancerous. Adenocarcinomas which evolve from the glandular tissue make up a total of 95% of colorectal cancers (American Cancer Society, 2005). In Malaysia, the colorectal cancer account for 14.2% and 10.1% for male and female respectively. It is the commonest cancer in male and the third commonest in women according to the National Cancer Registry (Lim and Halimah, 2004).

2.5 Nasopharyngeal Cancer (NPC)

Nasopharyngeal cancer occurs in the nasopharynx area which is located in the back of the nose toward the base of skull (American Cancer Society, 2006). The frequency the different types occurring vary according geographical area (American Cancer Society, 2006). According to the National Cancer Registry report, the nasopharyngeal cancer is the second most common cancer among men in Malaysia which is 8.8% of the total male cancers (Lim and Halimah, 2004). The male to female ratio for this cancer is 2.75 to 1 (Lim and Halimah, 2004).

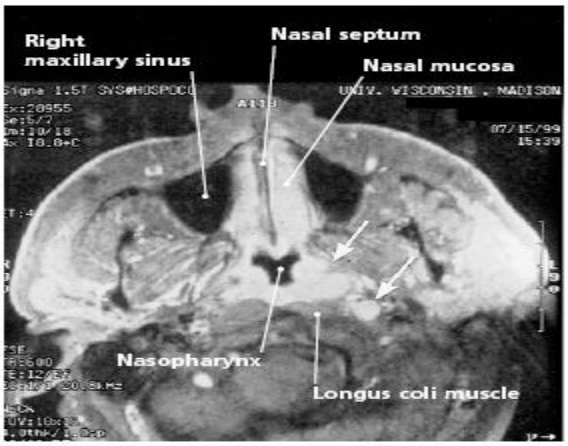

Figure 1: Sagittal magnetic resonance image in a patient with newly diagnosed squamous cell carcinoma of the nasopharynx. The arrows denote the primary tumor and a lateral retropharyngeal node metastasis. Taken from: www.Aafp.org/afp.

Basically, there are three types of NPC which are Type I, Type II and Type III NPC. Type I is the keratinizing squamous cell carcinoma, while Type II is the non-keratinizing carcinoma, whereas Type III is the undifferentiated carcinoma (American Cancer Society, 2006). Among the risk factors said to cause NPC is the consumption of salted-fish and other salt preserved food, occupational hazards like dust and smoke particles. Besides that, NPC is also said to be linked to the Epstein Barr virus (Her, 2001).

2.6 Oral Cancer

Oral cancer occurs in the oral cavity which includes the lips, the inside lining of the lips and cheeks (buccal mucosa), the teeth, the gums, the front two-thirds of the tongue, the floor of the mouth below the tongue, the hard palate, and the area behind the retromolar trigone (American Cancer Society, 2006). According to Peterson (2003), oral cancer prevails in men and is the eighth most common cancer worldwide. According to author in the World Oral Health Report in 2003, this cancer ranks amongst the three most common types of cancer in South Central Asia.

Leukoplakia and erythroplakia are abnormal areas in the mouth or throat which are usually white and red respectively. These may be a cancer or a precancerous condition called *dysplasia* (American Cancer Society, 2006). However, most oral cancers are squamous cell carcinomas which begin in the squamous cells that line of the oral cavity and oropharynx (American Cancer Society, 2006). Another type of oral cancer is the verrucous carcinoma which also is a type of low grade squamous cell carcinoma that rarely metastasizes but can deeply spread into surrounding tissue (American Cancer Society, 2006).

2.7 Tumor Susceptibility Gene 101 (*TSG101*) and Human Cancers

The *TSG101* have been linked with many types of tumours such as breast cancers (Wagner *et al.,* 1998; Balz *et al.,* 2002; Carney *et al.,* 1998), ovarian cancers (Balz *et al.,* 2002; Carney *et al.,* 1998), papillary thyroid carcinomas (Liu *et al.,* 2002), cervical carcinomas (Klaes *et al.,* 1999), prostate cancer (Sun *et al.,* 1997) and liver cancers (Chen *et al.,* 1999). However, in most studies done, genetic alteration such as somatic mutations and rearrangement are infrequent in *TSG101*. Most findings revealed the presence of aberrant splice forms of the *TSG101*. According to Wagner *et al.* (1998), most of the aberrant transcripts found were actually true alternative splice forms which were formed by exon skipping. Nevertheless, Wagner and co-workers (1998) did mention that the shorter transcripts reported (Lee and Feinberg, 1997) are probably real aberrant splice forms because it does not encode for any functional domain of the *TSG101* protein. There have been suggestions that the shorter transcripts are caused by splicing events at either existing intron to exon junctions which produce alternative spliced products (Wagner *et al.,* 1998) or cryptic splice donor and

acceptor sites within exons which causes aberrant splicing (Wagner *et al.*, 1998; Lee and Feinberg, 1997).

The *TSG101* has been a controversial gene because not only were aberrant transcripts and alternative splice forms found in cancerous cells, they are also found in normal cells (Klaes *et al.*, 1999; Carney *et al.*, 1998; Chang *et al.*, 1999; Lee and Feinberg, 1997; Xu *et al.*, 1998). However, there have also been reports where no aberrant transcripts or any mutations of the *TSG101* detected in the both the cancer cells and normal cells (Trink *et al.*, 1998). There has also been a proposed argument saying that the aberrant transcripts could actually also be PCR artifacts. However, Balz and colleagues (2002) refuted this statement because from their work, the aberrant transcripts were detected in the breast cancer patient, patient's mother who has breast cancer and not in the healthy daughter.

Besides that, the *TSG101* gene has also been known to be overexpressed in human cancers. In 2002, Liu *et al.* demonstrated by using immnunohistochemistry and *in situ* hybridization that *TSG101* was overexpressed in papillary thyroid carcinomas. In the study of differentially expressed genes using DNA microarray technology by Sim *et al*. (2006), the *TSG101* gene was consistently up-regulated in colorectal cancer. The authors also mentioned that this gene is listed as an up-regulated gene of colorectal cancers in the Cancer Gene Expression Database.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Total RNA

The total RNA for oral sample, bladder tumour samples and nasopharyngeal tumour and normal samples were obtaining from Chia Sze Wooi, Nur Diana Anuar and Joy Tan (personal communication; Immunology Laboratory, Dept of Molecular Biology, UNIMAS).

Table 1: RNA Samples obtained from the immunology lab and their classification.

Samples	Type of sample (normal/tumour)	Classification	Obtained from
Oral sample:			
1. P3	unknown	Not classified	Sarawak General Hospital
Bladder samples:		All samples are high grade transition cell carcinoma of the bladder. (according to SGH HPE reports)	Sarawak General Hospital
1. S14N	Normal		
2. S14T	Tumour		
3. S24T	Tumour		
4. S19T	Tumour		
5. S18T	Tumour		
6. S12T	Tumour		
Nasopharyngeal (NPC) samples:			
1. HS64	Normal	normal	Serian Hospital
2. HS59	Tumour	Type II	
3. HS60	Tumour	Type II	
4. HS65	Tumour	Type III	
Colon Samples			
1. CN	Normal	Normal (Healthy)	Chemicon
2. CT	Tumour	Adenocarcinoma	