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Identification of Tumor Suppressor Genes via Cell Fusion and Chromosomal Transfer

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1. Introduction

Loss of DNA or chromosomal deletion was frequently reported in different sporadic tumors, suggesting that those lost regions may contain putative tumor suppressor genes (TSGs). To map and isolate candidate genes from vast randomly lost areas, functional and complementary evidence is usually needed to define these areas to critical regions (CR). This is particularly important when one is dealing with sporadic cancers where clearly defined familial predisposition is present but high cancer risk families are not available for position cloning. A numerous studies have revealed extensive DNA deletions in nasopharyngeal carcinoma (NPC) and esophageal squamous cell carcinoma (ESCC). To identify candidate genes from these cancers, we used cell fusion and microcell-mediated chromosome transfer (MMCT) to introduce the whole chromosome or a chromosome fragment, into NPC and ESCC cell lines. Combined with other molecular approaches, we have successfully identified a number of novel TSGs on various human chromosomes.

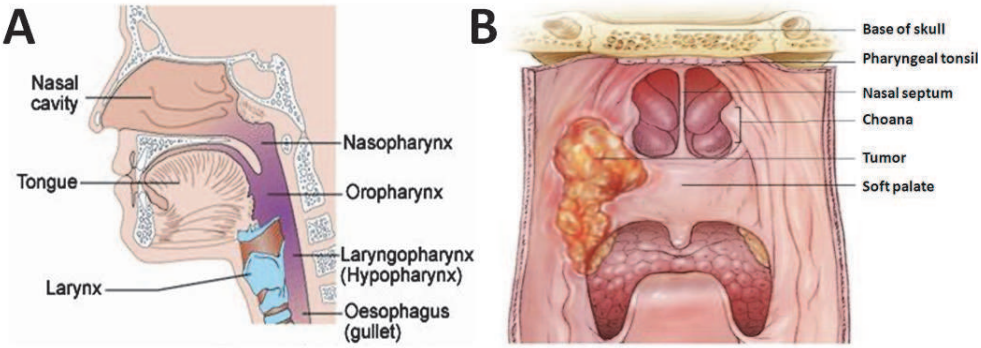
The nasopharynx is located behind the nasal cavity in the upper part of the pharynx (Fig. 1). NPC is a type of malignancy which arises from the epithelial cells in the nasopharynx. NPC is an unique cancer, which is commonly found in, Southeast Asia, North Africa, Middle East, and Arctic regions, but rare in most parts of the world (Jeyakumar et al 2006, Wei et al 2005). The esophagus is a tube, which is about 25 cm long, for the food passage from mouth to stomach (Fig. 2). Esophageal cancer (EC) is classified into two major histologic subtypes: squamous cell carcinoma and adenocarcinoma (Daly et al 2000). Esophageal adenocarcinoma (EAC) arises from the cells of glands responsible for producing mucous in the esophageal wall. The majority of the cases (>80%) are ESCC in Hong Kong, while EAC shows a climbing incidence in Western countries. EC varies greatly in geographical distribution; high-risk areas include north-central China in Henan and Shanxi (Holmes et al 2007, Qi et al 2005, Wu et al 2006).

2. Chromosome transfer in tumor studies

2.1 Somatic cell fusion and tumor suppression

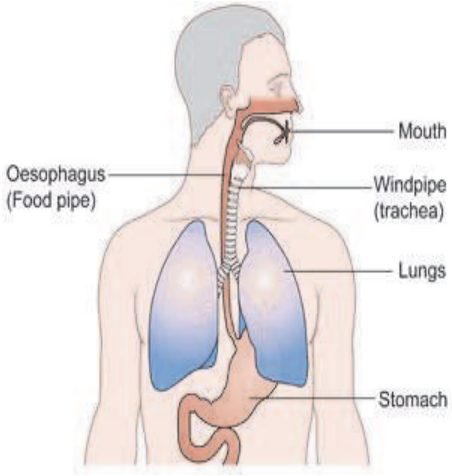
The theory for specific chromosomes contributing to tumor suppression was originally proven by somatic cell genetics (Harris et al 1969). In their study, Harris and colleagues fused the

mouse tumorigenic cells with non-tumorigenic cells to form a hybrid cell; some hybrid cells maintained the phenotypes of the parental cell lines and did not form tumors in the tumorigenicity assay. This suggests that non-tumorigenic cells contain genes, which are dominant and capable of suppressing the tumorigenic cell growth. The tumor suppression was further observed in human cell fusion experiments (Stanbridge, 1976 and 1992). The unstable tumorigenic hybrids showed loss of the selected chromosomes, resulting in the reemergence of its tumorigenicity properties. These findings suggested that some chromosomes may contain special regions, which can inhibit tumor formation, and thus provided the basis for further development of monochromosome transfer approaches to study the role of a particular chromosome in tumor development. These earlier experiments led to the hypothesis that the human genome might contain a group of genes suppressing tumor growth.



Adapted from Cancer Research UK and American Society of Clinical Oncology

Fig. 1. (A) Anatomy of human nasopharynx. (B) Location of nasopharyngeal carcinoma.



Adapted from Cancer Research UK

Fig. 2. Anatomy of human esophagus.

2.2 MMCT

The microcell-mediated chromosome transfer (MMCT) technique is a functional approach to investigate the tumor suppressive role of a specific chromosome or chromosome fragment in recipient cells. By using this approach, a single human chromosome is transferred into cancer cell lines. Transfers of exogenous chromosomes are tested for their functional ability to complement existing defects present in cancer cells. In this approach, a microcell donor, which contains a human chromosome of interest, is used as a donor to transfer the selected chromosome into tumor cells. Microcells, presumably with one chromosome within a nuclear envelope and plasma membrane, are generated after disruption of the cytoskeleton of the donor cells. The microcells are then fused to a malignant cancer cell line to establish stable microcell hybrids (MCHs) (Anderson et al 1993, Fournier et al 1977a, Stanbridge 1992). DNA genotyping and fluorescence in situ hybridization (FISH) are used for verification of a successful transfer. An in vivo nude mouse assay is then used to assess the tumorigenic potential of MCHs (Cheng et al 1998, Cheng et al 2000, Cheng et al 2002, Cheng et al 2003, Cheng et al 2004, Cheung et al 2009, Lung et al 2008a).

2.3 Advantages of MMCT approach in TSG studies

For the identification of TSGs contributing to the human cancers, initial efforts used the positional cloning of target regions defined by linkage analysis of pedigrees in hereditary cancer studies. The successful finding of the retinoblastoma gene, RB, is a good example (Friend et al 1986). However, the vast majority of the human cancers develop sporadically and involve numerous multiple gross deletions of chromosomal regions known as loss of heterozygosity (LOH). MMCT is a functional complementation approach useful for the identification of recessive-acting TSGs in sporadic cancers (Murakami 2002). Gene transfer approaches into human cancer cells may involve whole cell fusion, microcell fusion for intact or fragmented chromosomes, spheroplast fusion of yeast artificial chromosome (YAC), lipofection of bacteriophage P1, P1-derived artificial chromosome (PAC), bacterial artificial chromosome (BAC), or plasmids carrying the target genes, etc (Table 1) (Murakami 2002).

One of the advantages of the MMCT method is the transfer of a single copy of a chromosome allows strict control of dosage effect. If the transfer method is based on lipofection, it is impossible to control the copy number of DNA integrations into the genome. Compared to other commonly used artificial over-expression systems, the genes transferred by MMCT are expressed under the control of their endogenous promoters and enhancers and are regulated in their native environment. Thus, MMCT provides an ideal method for studying gene expression closely mimicking physiological levels and regulation controls under its native environment. Although the gene transfer by lipofection is convenient, the biological significance of functional studies of genes by the introduction of an expression vector should be carefully evaluated because the ectopic expression level of genes are not always equivalent to that found in the normal or tumor tissues, but are actually extraordinarily high in transfectants. An important issue to be addressed in the functional study of TSGs is whether the growth suppression resulting from exogenous gene over-expression by artificial procedures is a non-specific cytotoxic or cytostatic effect rather than a specific effect due to the expression of the gene itself.

In the second step of the functional complementation cloning of TSGs, the *in vivo* tumorigenicity assay in the immunologically deficient mice is an indicator of the malignant

phenotype of cancer cells. The nude mouse tumorigenicity assay allows us to identify genes that fit the ultimate definition of a TSG, which requires functional evidence for the suppression of the malignant phenotype of cancer cells. The functional identification of TSGs in nude mouse assay can exclude the non-specific cytotoxic or cytostatic effects introduced by artificial gene expression. It also identifies a new category of molecules that are not cytotoxic *in vitro*, but are involved in the signaling cascades in different biological processes that hallmark cancer development such as the cell adhesion and sustained angiogenesis, inflammatory responses, evading immune destruction, and the tumor microenvironment.

Vector	Method	Length of the insert	No of genes	Promoter	Position effect	DNA copy number	References
Cell	Whole cell fusion	3 Gb	~30,000	Endogenous	-	2	(Murayama et al 1965) (Harris et al 1969)
Chromosome	Microcell fusion	~ 150 Mb	~1,500	Endogenous	-	1	(Fournier et al 1977b) (Weissman et al 1987) (Koi et al 1989)
Chromosomal fragment	γ-Irradiation + microcell fusion	2-20 Mb	20-200	Endogenous	-	1	(Dowdy et al 1990, Koi et al 1993), (Murakami et al 1995)
YAC	Spheroplast fusion	100-1600 kb	1-20	Endogenous	+ < -	1	(Pavan et al 1990b) (Pachnis et al 1990, Wada et al 1994), (Murakami et al 1998b)
Fragmented YAC	Homologous recombination + Spheroplast fusion	100-500 kb	1-10	Endogenous	+ / -	1	(Pavan et al 1990a), (Murakami et al 1998a)
P1, PAC, BAC	Lipofection	80-200 kb	<10	Endogenous	+ > -	>1-10	(Todd et al 1996)
Plasmid	Lipofection etc.	<5 kb (cDNA)	1	Artificial	+	>1-10	Numerous report

Adapted from Murakami (2002).

Table 1. Gene transfer methods into mammalian cells.

3. How to identify a TSG from a pool of genes?

3.1 MMCT

An intact single chromosome can be introduced into the recipient cell line using MMCT techniques. The establishment of mouse donor cells with a single copy of human chromosome is described previously (Fournier and Ruddle 1977a, Saxon et al 1985, Anderson and Stanbridge 1993). Figure 3 briefly outlines the procedures of MMCT. Donor cells were seeded on day 1 and incubated with 0.04 to 0.1 μg/ml colcemid for 48 to 56 hours to arrest cells in metaphase. Recipient cells were seeded on day 2 with the cell confluence reaching 80-90% the next day. Enucleation of the donor cells was achieved by centrifugation at 13,000 rpm for 65 to 70 min at 34°C with 10 μg/ml cytochalasin B (Sigma, MO, USA). The heterogeneous microcell mixture was then filtered through successive polycarbonate filters of 8/5/3 μm pore sizes (Whatman, Middlesex, UK) to remove large microcells and other cell debris. Microcells were pelleted by centrifugation and resuspended thoroughly in 1 ml DMEM containing 50 μg/ml phytohemagglutinin-p (PHA-P). The recipients were rinsed in PBS once and the microcell/PHA-P suspension was added to the recipient cells and incubated for 20-40 min. Microcells were fused to the recipient cell with 1-2 ml ice-cold PEG-1000. The treated cells were rinsed with PBS four times and fed with medium. The selection medium [hypoxanthine, aminopterin, and thymidine (HAT) + G418] was added on day five. After about three-four weeks selection, only the recipient cells harboring the transferred chromosome containing the neomycin gene on the exogenously transferred chromosome survive; potential hybrids were picked and expanded for further analysis.

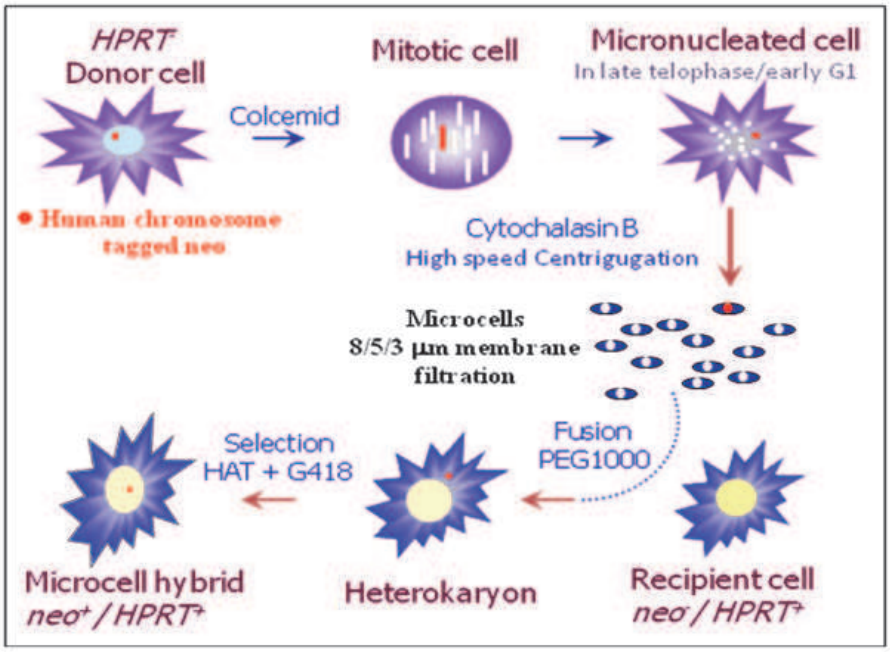


Fig. 3. Schematic diagram of MMCT.

3.2 Confirmation of successful chromosome transfer

The MCHs were subjected to DNA extraction and confirmation for the absence of mouse DNA contamination by DNA slot blot hybridization. Then the MCHs were subjected to microsatellite typing and fluorescence *in situ* hybridization (FISH). For microsatellite analysis, PCR products of microsatellite markers that amplified fragments with various numbers of repeat units are separated by gel or capillary electrophoresis. Microsatellite typing is a fast, simple, and reliable method to identify whether the donor allele has been transferred into the recipient cancer cell line. The high heterozygosity rate of microsatellite markers is used to demonstrate hybrid patterns in MCHs versus the recipient and donor cells to verify successful transfer of the donor human genetic materials into the human cancer cell line at the molecular level after MMCT.

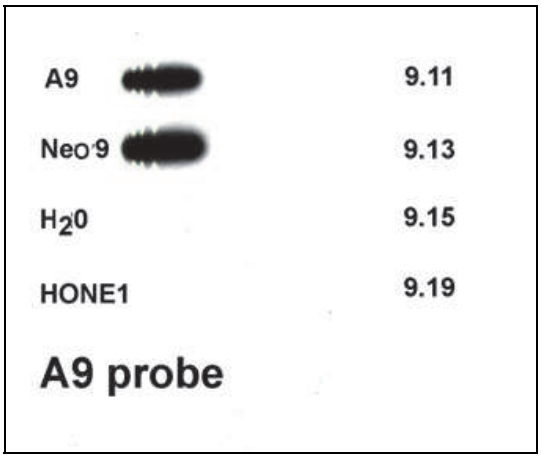


Fig. 4. DNA Slot blot analysis of chromosome 9 MCHs. The DNA of mouse A9 cells was used as a probe. The A9 and donor Neo 9 cells were used as positive controls for presence of mouse DNA, while the recipient cell line, HONE1 was used as a negative control for absence of mouse DNA. All MCHs was found to have no mouse DNA contamination.

3.2.1 DNA slot blot hybridization

The contamination of mouse DNA in MCHs was excluded by slot blot hybridization (Fig. 4). Five micrograms of genomic DNA of recipient cell lines, HONE1, microcell donor, MCHs, and A9 in 6X SSC (0.9 M NaCl and 90 mM Na Citrate, pH 7.4) were transferred onto a HybondTM N nylon membrane (Amersham Biosciences, Uppsala, Sweden) with a BIO-DOT® SF slot blot apparatus (BioRad, CA, USA). The samples on the membrane were denatured for 10 min with a denaturing solution (1.5 M NaCl and 0.5 M NaOH) and then neutralized in 1 M NaCl and 0.5 M Tris-Cl, pH 7 for 5 min. The membrane was dried and cross-linked by UV on an UV transilluminator (Stratagene, CA, USA). A Gene ImagesTM AlkPhos DirectTM Labelling and Detection System (Amersham Biosciences, Uppsala, Sweden) was used according to manufacturer's protocol. In brief, a total of 100 ng of A9 DNA was denatured by heat for 5 min. The probe was labeled by covalently linking to alkaline phosphatase by incubating at 37°C for 30 min with crosslinker solution and reaction buffer. The blot was prehybridized with 0.125 ml/cm2 hybridization buffer containing 12%

w/v urea, 0.5 M NaCl and 4% blocking reagent at 55°C for at least 15 min. The labeled probe was added into hybridization buffer and hybridized to the membrane containing samples at 55°C overnight. The blot was then washed twice with 55°C primary washing buffer (2 M Urea, 0.1% w/v SDS, 50 mM Na phosphate pH 7, 150 mM NaCl, 1 mM $MgCl_2$, 0.2% w/v blocking reagent) for 10 min with gentle agitation. The blot was then washed twice with secondary wash buffer (50 mM Tris-Cl, 100 mM NaCl, 2 mM $MgCl_2$, pH 10) at room temperature for 5 min. A chemiluminescent signal was generated by adding 40 µl/cm² CDP-Star detection reagent (Amersham Biosciences, Uppsala, Sweden) and detected on X-ray film.

3.2.2 Microsatellite typing

Microsatellite typing was used in genomic analysis in this study (Fig. 5). The polymorphic markers, showing a high heterozygosity frequency, were used. PCR was performed to amplify genomic regions of particular markers. PCR reactions were carried out with 100 ng of DNA template, 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA), 0.3 µM of each fluorescence-labeled primer. PCR products were injected into semi-automated ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems, CA, USA) for capillary electrophoresis and analysis by Genotyper software. All markers were labeled with 6-FAM/VIC/NED fluorescence dye and have annealing temperatures of 55°C.

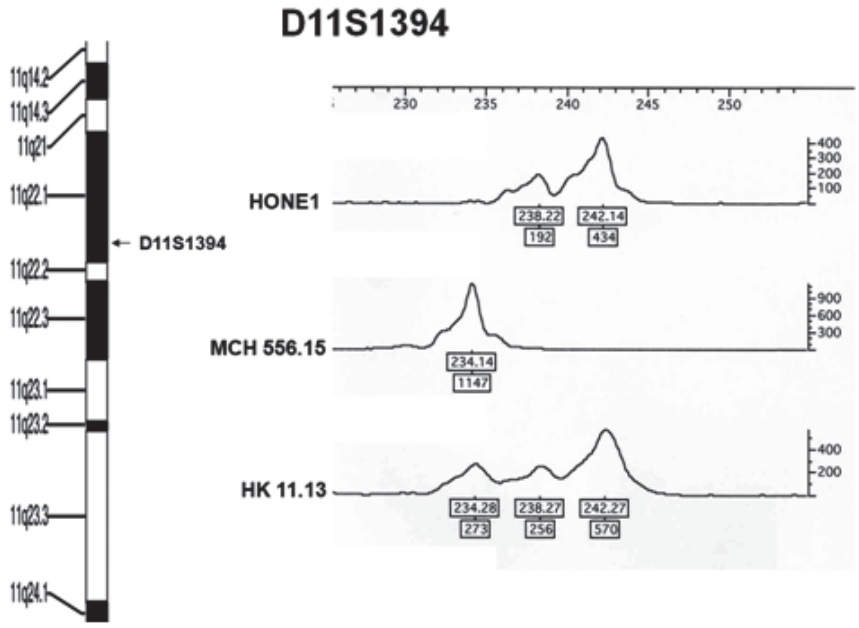


Fig. 5. PCR microsatellite typing of a representative donor, recipient, and hybrid cell line. The recipient HONE1, donor MCH556.15, and MCH HK11.13 cell lines were analyzed with the D11S1394 primers. The combined peak patterns of the hybrid cell line show successful transfer of chromosome 11 in recipient cells.

3.2.3 Whole chromosome painting FISH

The confirmation of the presence of an extra copy of intact human chromosome in the hybrids was performed by whole chromosome painting FISH analysis. To obtain metaphase cells, logarithmic growth phase cells were treated with 0.05 μg/ml colcemid for 4-6 hours. After harvesting the cells by centrifugation at 4000 rpm for 5 min, they were subjected to hypotonic treatment (0.075 M KCl) for no more than 25 min at 37°C. Cells were fixed with methanol/acetic acid (3:1) overnight and then dropped onto glass slides. The whole chromosome paint (WCP) probes were used and they are a mixture of DNA sequences specific to a particular chromosome that are directly labeled with one of the Vysis™ fluorophores. The slides were denatured at 73°C for 5 min [70% (v/v) formamide, 2 X SSC (pH7), 0.1 mM EDTA]. Slides were dehydrated for 1 min in 70%, 85%, and 100% ethanol successively. Probes were hybridized to the slides in LSI/WCP® hybridization buffer at 37°C in dark for overnight. The slide was washed at 73°C in washing solution I (0.4 X SSC, 0.3% NP40) for no more than 2 min, followed by washing with washing solution II (2 X SSC, 0.1% NP40), and counterstained with DAPI. Fluorescence signals were captured on an Olympus BX51 fluorescence microscope with the Spot software.

3.3 *In vivo* nude mouse tumorigenicity assay and establishment of tumor segregants (TSs)

After successful chromosome transfer, MCHs containing an exogenous chromosome were obtained and the tumorigenicity assay was then performed. The tumorigenicity of the each NPC/ESCC cell line was investigated by subcutaneous injection of 1 X 10^7 cells into 6-8 week old female athymic BALB/c *nu/nu* mice. This assay has been repeatedly and successfully used for assessing the degree of tumor suppression in MCH populations (Goyette et al 1992, Saxon et al 1985, Weissman et al 1987). For each tested cell line, at least six sites were injected on three mice (two sites per animal). The animals were monitored regularly for tumor formation and palpable nodules were measured weekly using calipers. After a long period of incubation in the nude mice (>12 weeks), if notable tumors formed, they were surgically removed aseptically and then washed with PBS, followed by mincing the tissues as small as possible in a petri dish. The minced tissue was covered with 5 ml medium and left without any disturbance for at least three days. Medium was withdrawn and 10 ml of fresh medium was added. The cells were then harvested and were the TSs/revertants used in further analysis with their matched MCHs.

3.4 TS analysis

The TSs show a restoration of tumorigenicity in the tumorigenicity assay by elimination of critical regions (CRs) associated with tumor suppression (Cheng et al 2002, Cheung et al 2009). Loss of donor genetic materials detected by microsatellite typing (see Section 3.2.2) was considered significant, when the intensity of the donor to recipient allele ratio in a TS versus its corresponding MCH parental cell line, was equal to or less than 0.8. The significance of these losses was simultaneously verified by bacterial artificial chromosome (BAC) FISH. Furthermore, the differential gene expression profiling between the tumor-suppressive MCH cell lines versus their derived TSs and the highly tumorigenic recipient cell line was revealed by oligo microarray analysis. The BAC FISH and oligo microarray analyses will be discussed in detail.

3.4.1 Bacterial artificial chromosome (BAC) FISH

The BAC DNA probes were prepared by labeling the isolated BAC DNAs by nick translation. In brief, BAC clones were incubated in 5 ml LB medium containing 12.5 µg/ml chloramphenicol at 37°C overnight. Bacterial cells were pelleted down and resuspended in 200 µl of ice-cold alkaline lysis solution I. Four hundred microliters of freshly prepared alkaline lysis solution II were added, mixed by gentle inversion several times, and placed on ice for no more than 5 minutes. A total of 300 µl of ice-cold solution III was added, and the mixture was put on ice for 5 minutes, followed by centrifugation at maximum speed for five minutes at 4°C. Supernatant was transferred into a fresh 1.5 ml tube containing 900 µl of isopropanol at room temperature and mixed by gentle inversion. The precipitated DNA was collected by centrifugation at maximum speed for five minutes at 4°C; supernatant was discarded and the pellet was rinsed with 1 ml of 75% ethanol. The tube was centrifuged for 5 min at room temperature and ethanol was removed. The DNA pellet was air-dried and dissolved in 50 µl TE.

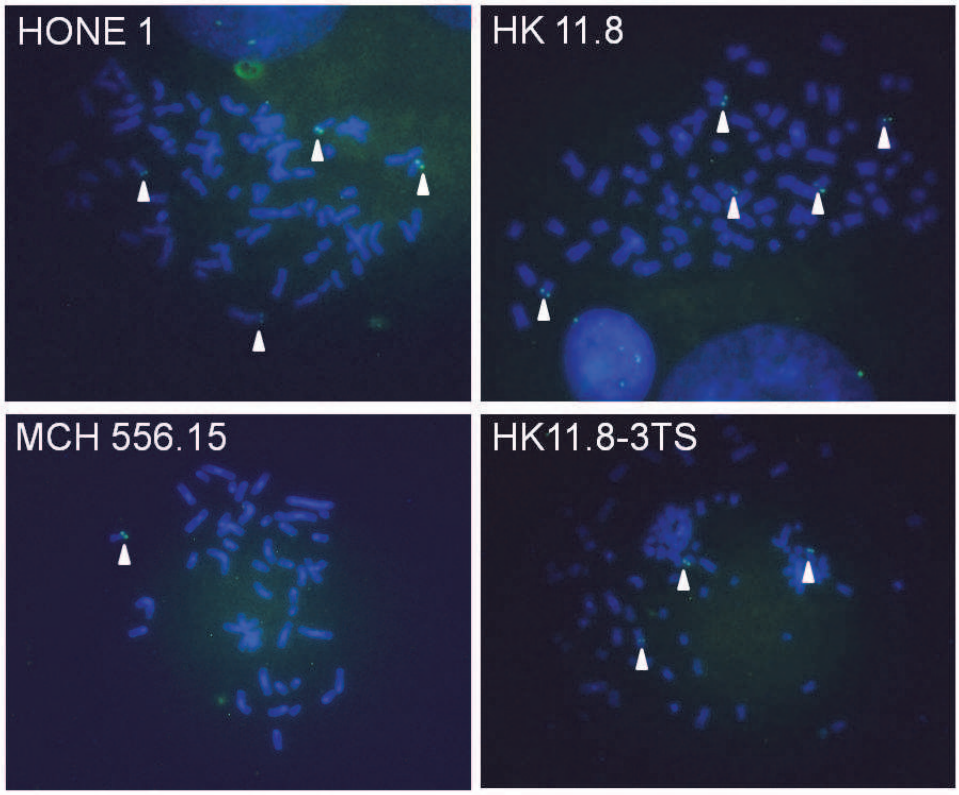


Fig. 6. FISH analysis with BAC CTB-12N1 probe in recipient HONE1 cells, chromosome 11 donor MCH556.15 cells, hybrid HK11.8 cells, and tumor revertant HK11.8-3TS cells. Thirty metaphases on average were observed for each cell line. Four copies of the BAC probe are observed in HONE1 cells, one in the donor cell line, 5 in the hybrid HK11.8 and 3 in the HK11.8-3TS. Arrows (▲) indicate BAC hybridization.

A total of 500 ng of BAC DNA was labeled with 10 µM SpectrumGreen/Orange TM-dUTP (Vysis, IL, USA), nick translation buffer (Vysis, IL, USA), 10 µl nick translation enzyme (Vysis, IL, USA) at 14°C for 16 hours and stopped at 70°C for 10 minutes. The probe was precipitated with 1 µg of COT-1 DNA (Invitrogen, CA, USA) and 2 µg salmon sperm DNA (Invitrogen, CA, USA), 4 µl water, 0.1 volume of 2 M sodium acetate, 2.5 volumes 100% ethanol and centrifuged at maximum speed for 30 min at 4°C. The supernatant was removed. The pellet was air-dried and resuspended in 3 µl water and 7 µl LSI/WCP hybridization buffer. Procedures for the hybridization and detection were the same as that used for the whole chromosome painting FISH. Representative BAC FISH results are shown in Figure 6.

3.4.2 Oligonucleotide microarray

In order to investigate the differentially expressed genes of the tumor-suppressive MCHs versus their matched tumorigenic TSs, oligonucleotide microarray analysis was performed; a TSG is presumably up-regulated in MCHs and down-regulated in TSs (Robertson et al 1997). Therefore, genes with that expression profile are potential TSGs in NPC. This method can also help to identify candidate genes that are downstream of the functional pathways. The 19K or 28K oligonucleotides were spotted on glass slides using a custom-built microarray spotter at the Genome Institute of Singapore. The incorporation of Cy3 and Cy5 to cDNAs, competitive hybridizations, and processing of array images were performed as described (Lin et al 2004).

In brief, a total of 20 µg of total RNA was mixed with 1 µg oligo dT and denatured at 65°C for 5 min. Reverse transcription was done by using 1X first strand buffer, 4 µl of 0.1 M DTT, 2 µl 20X low dT/aa-dUTP mix (1 M dATP, 1 M dGTP, 1 M dCTP, 200 mM dTTP, and 800 mM amminoallyl dUTP), 100 unit of RNase OUT inhibitor (Invitrogen, CA, USA), and 400 units of SuperScript II reverse transcriptase (Invitrogen, CA, USA) and incubated at 42°C for 60 min. Another 200 units of SuperScript II reverse transcriptase was added to the mixture and then it was incubated at 42°C for 60 min. Five microliter of 500 mM EDTA (pH 8) and 10 µl of 1M NaOH were added to the reaction mixture and incubated at 65°C for 45 min, followed by adding 25 µl of 1 M Tris-Cl (pH 7.5) and 400 µl ddH_2O and purified on a YM-30 column (Millipore, MA, USA). One microliter of 500 mM $NaHCO_3$ (pH 9) and fluorescent dye, Cy3 or Cy5 (GE Health Care Life Sciences, NJ, USA), were added to 9 µl of the cDNA for dye incorporation at room temperature for 1 hour.

The chips, which contain synthesized 60-mers of 19K or 28K oligonucleotides, were prehybridized by using DIG Easy Hyb (Roche Diagnostics, Basel, Switzerland) at 42°C for one hour. Four microliter of 4 M hydroxylamine (Sigma, MO, USA) was added to the labeled cDNA at room temperature for 15 min. Thirty-five microliter of 100 mM NaOAc (pH 5.2) was added and mixed with the paired sample labeled with Cy3 and Cy5. The reaction mixtures were purified on YM-30 columns, containing 40 µg of herring sperm DNA (Invitrogen, CA, USA) and 22 µl DIG Easy Hyb were added, followed by denaturing at 65°C for 5 min. The cDNA probe was hybridized onto slides and incubated in the MAUI hybridization chamber (BioMicro Systems, UT, USA) at 42°C for 16 hours. After hybridization, slides were washed with 2X SSC + 0.1% SDS for 30 s, 1X SSC for 30 s and 0.2X SSC for 30 s and 0.05X SSC for 5 s. The slides were then scanned with GenePix 3100 microarray scanner and analyzed by GenePix Pro 4.0 and 5.0 software (Axon Instruments, CA, USA). The results were uploaded to the database from Genome Institute of Singapore

(GIS MAdb, http://gismadb.gis.a-star.edu.sg). The hierarchical clustering was performed by CLUSTER program and the results were viewed by the TREE VIEW program (M. Eisen; http://rana.lbl.gov/EisenSoftware.htm). Stringency was set so that only data sets with expression ratios higher than 1.4 in hybrids/recipient cells and ratios less than 0.6 in hybrids/TSs for both duplicates were selected for further studies.

3.5 Methylation study of candidate tumor suppressor genes

Promoter hypermethylation is a major mechanism for silencing TSG expression. Promoter hypermethylation only occurs at cytosine (C), which is located on the 5′ end of guanine (G) of promoter CpG islands. Addition of a methyl group to cytosine by methyltransferase blocks the binding of the activator or inhibitor to the promoter sequence and consequently there is transcriptional silencing of the gene. By using 5-aza-2′-deoxycytidine, which can demethylate the methyl groups in the CpG islands, gene expression can be restored. Using this approach, methylation status of genes of interest may be elucidated. Besides using the 5-aza-2″-deoxycytidine, bisulfite genomic sequencing (BGS) and methylation-specific PCR (MSP) are other important methods used to analyze promoter hypermethylation. In the bisulfite treatment, the genomic DNAs are modified. All of the unmethylated cytosines are converted to uracils, while the methylated cytosines remain unchanged. By analyzing the promoter sequences and using sequence-specific primers, the promoter hypermethylation status can be determined.

3.5.1 Bisulfite treatment

Genomic DNA was treated with sodium metabisulfite to analyze the promoter hypermethylation status. Two micrograms of DNA were dissolved in 10 µl of water, followed by adding 1.1 µl of 3N NaOH and incubation at 37°C for 15 min. Then 104 µl of urea/metabisulfite solution (6.24 M urea and 2 M sodium metabisulfite; Sigma, MO, USA), 6 µl of 10 mM hydroquinone (Sigma, MO, USA) and 100 µl of mineral oil were added into the mixture and incubated at 55°C for no more than 15 hours. The mixture was then purified by QIAquick PCR purification Kit (Qiagen, CA, USA) according to the manufacturer's protocol and eluted with 200 µl elution buffer. The purified DNA was denatured in 23 µl of 3N NaOH at 37°C for 15 min. The DNA was precipitated by adding 2 µl of tRNA (10 µg/µl; USB, OH, USA), 50 µl of 10 M ammonium acetate, and 500 µl of 100% ethanol, and stored at -20°C for 30 min. The mixture was centrifuged at 12K rpm at 4°C for 20 min; the supernatant was removed. The DNA pellet was washed with 500 µl of 70% ethanol and centrifuged at full speed at 4°C for 20 min. The DNA pellet was air-dried and dissolved in 100 µl of 10 mM Tris-Cl (pH 8.5) solution.

3.5.2 Bisulfite genomic sequencing (BGS) and methylation-specific PCR (MSP)

BGS and MSP PCR reactions were carried out with 5 µl of bisulfite-treated DNA template, 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA), 0.3 µM of primers. For the BGS, PCR products were then cloned into the TA cloning pMD18T Simple vector (TaKaRa Biotechnology, Dalian, China) and transformed into competent cells. Mini-preparation was performed to extract the plasmid DNA. For each examined cell line, five individual clones were checked to confirm the promoter hypermethylation status. The plasmids were then sequenced by using pMD18T7 forward and reverse sequencing primers and BigDye 3.1 terminator. Signals were

detected using an ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems, CA, USA). The bisulfite sequencing and MSP primers were designed by the MethPrimer (www.urogene.org/methprimer) guide (Li et al 2002).

3.5.3 5-Aza-2'-deoxycytidine treatment

The re-expression of the candidate genes silenced by promoter hypermethylation was investigated using a demethylation reagent. Cells were treated with 5 μM 5-aza-2'-deoxycytidine (Sigma, MO, USA) for five days; freshly diluted drug was changed daily. After 5 days treatment, the cells were harvested for RNA extraction.

3.6 Other functional assays we use to study the tumor suppressive phenotypes

In order to examine the tumor suppressive phenotypes of NPC and EC cells expressing a candidate TSG, various functional assays were performed according to the major hallmarks of cancer (Hanahan et al 2011). Simple proliferation, cell cycle, colony formation, and soft agar assays are routinely performed to assess the inhibitory effects of the transgenes on cell growth ability in normal culture conditions, the change of cell cycle status, the ability of colony formation from a single cells, and the cell growth potential in an the anchorage-independent environment. In addition, the migration, invasion, and angiogenic properties of the tumor suppressive transfectants were also examined. Some of those functional assays will be discussed in detail.

3.6.1 Three-dimensional matrigel culture

Matrigel contains a gelatinous protein mixture of extracellular matrix proteins such as laminin and collagen, which recapitulates the natural environment of tumor cells for better investigation of the functional impact of a candidate TSG. We aim to see whether there is any change in the numbers, sizes, and morphologies of colonies of transfectants with the transgenes versus the vector-alone, when grown in matrigel. In brief, matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA) was thawed on ice and 100 μl was coated as a bottom layer onto each well of a 24-well cluster plate. Subsequently, a total of 5000 cells resuspended in 0.5 ml growth medium was seeded on top of this bottom matrigel layer. After 2 weeks, images were captured using an inverted light microscope (Nikon TMS, Ontario, Canada) at 20X magnification.

3.6.2 Cell migration and invasion assays

The cell migration and invasion abilities of cells were determined using a micropore chamber assay (BD Biosciences, San Jose, CA, USA). The chamber used for migration study consists of an 8 μm pore size PET membrane at the bottom of the insert, while the chamber used for invasion study is a migration chamber coated with a thin layer of matrigel basement matrix. The migration and invasion abilities of each clone were determined by the number of cells passing through the membrane and the matrigel basement matrix, respectively.

3.6.3 HUVEC tube formation assay

The tube formation assay is an *in vitro* angiogenesis assay to assess the tube formation ability of vascular endothelial cells. Briefly, the Human Umbilical Vein Endothelial Cell

(HUVEC) was cultured in collagen pre-coated TC dishes. The conditioned medium of each cell line was collected by incubating the cells with DMEM/RPMI serum-free medium for 24 hours. The 96-well plate pre-coated with 50 µl growth factor-reduced Matrigel (BD Bioscience, San Jose, CA, USA) per well was used. A total of 4 x 10^4 HUVEC cells resuspended in 100 µl conditioned media supplemented with 1% serum were seeded in each well. The HUVEC cells were then incubated at 37°C for 6 hours to allow the formation of tube-like structures. The tubes formed were captured under microscopy and the total tube length formed by each sample was measured by Spot software (Diagnostic Instruments Inc, Sterling Heights, MI, USA).

3.6.4 Matrigel plug angiogenesis study

In vivo angiogenesis study was performed by matrigel plug angiogenesis analysis. A total of 5 x 10^6 cells mixed with 50 µl DMEM and 250 µl ice-cold matrigel was injected into nude mice subcutaneously to allow gel plug formation. Each sample was injected into one flank of five female athymic nude mice. The matrigel mixed with the cells polymerizes to form a solid gel plug, which allows cell growth and blood vessel formation. After inoculation for 7 days, the matrigel plug was excised, fixed with formalin, embedded in paraffin, sectioned, and mounted onto slides. The slides were stained with hematoxylin and eosin (H&E) for histological observation. The blood vessels formed were stained with monoclonal antibody. The slide was scanned and the signal was quantified by ImageScope v10 software (Aperio, Vista, CA, USA).

3.6.5 Human angiogenesis antibody array

The angiogenesis-related proteins excreted from the TSGs versus the vector-alone transfectants were detected by the human angiogenesis antibody array (RayBiotech, Norcross, GA, USA). The array included two membranes pre-coated with 43 angiogenesis cytokine antibodies. The conditioned media were obtained as described in Section 3.6.3. After blocking and complete washing, the membranes were hybridized with biotin-conjugated antibody at room temperature for 2 hours, followed by hybridizing with HRP-conjugated streptavidin at room temperature for 2 hours. The membranes were then washed again and hybridized with the detection buffer. The signals were exposed to a X-ray film and quantified by Quantity One® software (Biorad, Hercules, CA, USA).

4. Examples of candidate TSGs studied via MMCT and other functional approaches

The technique of microcell fusion to transfer single normal chromosomes was used as a functional assay for the TSG activity (Goyette et al 1992, Saxon et al 1986, Weissman et al 1987). This has been particularly useful in confirming TSG functions associated with specific chromosomes, where the mapping location is suspected, but no candidate TSG has been cloned. In addition, by transferring chromosomes possessing interstitial deletions, it has been possible to map the location of TSGs more precisely (Dowdy et al 1991).

The analysis of tumor biopsy specimens, xenografts, and derived cell lines have identified an extensive chromosomal alterations in human genome, including those derived from chromosomes 1, 3, 9, 11, 12, 13, 14, 16, and 17 (Huang et al 1989, Mitelman et al 1983), Consistent with Knudson's "two-hit" theory, studies of chromosomal loss or deletion may

provide useful clues for identification of critical genes involved in inherited cancers. However, it should be appreciated that the limitations of deletion or loss studies in sporadic cases may miss critical regions for several reasons. First, the minimally deleted regions may be obscured when all of the genome shows some degree of LOH, which is common in many sporadic tumors. Second, vast evidence has shown that some TSGs, notably *p16* are silenced or imprinted by epigenetic events such as methylation, irrespective of their LOH status in tumors. Third, LOH was studied in primary tumors with karyotypic complexity, gene dosage changes, and contamination by normal cells. Finally, although LOH is commonly detected in practically all types of human cancers, neither LOH nor CGH (comparative genomic hybridization) studies provide functional evidence. To map novel TSGs, functional and complementary approaches are needed for distinguishing a critical region from extensive randomly lost areas. Chromosome 3 was the first chromosome used for the functional studies of tumor suppression in NPC. In this study we used a series of intact and deleted copies of human chromosome 3 derived from normal cells, with discrete interstitial deletions in the *p* arm, for transfer into the tumorigenic NPC HONE1 cell line. By using the MMCT approach, we successfully transferred these chromosome 3 fragments into HONE1 cells and localized a tumor suppressive region on this chromosome. Comparison of the tumorigenic potential of the MCHs containing these exogenous chromosome 3 fragments identified chromosome 3p21.3 as the first tumor suppressive region in NPC (Cheng et al 1998). This area was subsequently confirmed to harbor several TSGs associated with the development of NPC and other common cancers (Lerman and Minna 2000, Lo *et al* 2001, Yau *et al* 2006, Hesson *et al* 2007). Using these functional approaches, we also investigated known and candidate TSGs in other chromosomes, including chromosomes 9, 11, 13, 14 and 17. These chromosomes contain important TSGs, such as *p16, RB* and *p53* and a number of newly-identified TSGs. However, we only introduce a few of examples of these studies in this chapter due to the space limitation. (Cheng *et al*, 2000, 2002, 2003 and 2004, Ko et al 2005 and 2008, Lung *et al* 2008a and 2008b, Cheung *et al* 2009, Lo *et al* 2007 and 2010)

4.1 Chromosome 3: *ADAMTS9*

In a subsequent study, an intact and two truncated human chromosomes 3 obtained from the same panel of chromosome 3 donor cells, were transferred into the highly tumorigenic ESCC SLMT-1 cell line. Similarly, the ability of these transferred chromosomes to functionally complement defects in the ESCC cell line was assessed by examining the impact of this transfer on tumorigenic potential in nude mice. PCR-microsatellite and BAC FISH analyses were used to narrow down and identify the CR associated specifically with tumor suppression. A 1.61 Mb CR located between markers D3S1600 and D3S1285 was found to be necessary for the tumorigenic suppression of ESCC. These findings further suggest that the CR present in the exogenous chromosomes contains functional tumor suppressive elements. In the study, we identified a candidate TSG, *ADAMTS9*, and one non-coding RNA, ENST351926 mapping to 3p14.2, which are located in the chromosome 3 CR of ESCC. The expression of *ADAMTS9* in tumor suppressive MCHs was confirmed by reverse transcription (RT)–PCR. The positive expression of the gene was observed in all tumor suppressive MCHs, but was not found in tumorigenic MCHs and TSs, strongly suggesting that *ADAMTS9* plays an important role in tumor suppression. The pseudogene is located upstream of the *ADAMTS9* promoter region and whether it can serve as a riboregulator or gene expression regulator remains to be determined (Lo et al 2007).

4.1.1 *ADAMTS9 (A disintegrin-like and metalloprotease with thrombospondin type 1 motif 9)*

As described, using a functional genomic mapping approach, we identified a CR for tumor suppression at 3p14.2 and discovered the important role of A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif 9 (ADAMTS9), a gene previously mapped to this region (Clark et al 2000) in ESCC (Holmes and Vaughan 2007). ADAMTS9 encodes a member of a large family of 19 metalloproteases involved in maturation of precursor proteins, extracellular matrix remodeling, cell migration, and inhibition of angiogenesis (Apte 2004, Porter et al 2005). Although the related matrix metalloproteases and ADAM proteases have been clearly implicated in tumor progression and angiogenesis, the role of ADAMTS proteases in cancer is less clearly defined. ADAMTS1 was first identified as an anti-angiogenic molecule (Vazquez et al 1999), and shown to have anti-tumor effects. Recently, methylation studies identified another family member ADAMTS18 as crucial in several human cancers including NPC and ESCC (Jin et al 2007).

As mentioned in the previous section, *ADAMTS9* was identified as one of the differentially expressed genes in these non-tumorigenic MCHs and their matched TS cell lines in our previous study (Lo et al 2007). It was suggested that ADAMTS9 is associated with tumor suppression in human esophageal cancer. Promoter hypermethylation contributes to ADAMTS9 gene silencing in ESCC (Lo et al 2007, Lung et al 2008b). However, the functional impact of ADAMTS9 on cancer development had not been explored. In the follow-up study, we evaluated the hypothesized anti-angiogenic and tumor suppressive functions of ADAMTS9 in ESCC, by stringent tumorigenicity and matrigel plug angiogenesis assays (Lo et al 2010). ADAMTS9 activation suppressed tumor formation in nude mice. In vivo angiogenesis assays revealed a reduction in microvessel numbers in gel plugs injected with tumor-suppressive cell transfectants. Similarly, conditioned media from cell transfectants dramatically reduced the tube-forming capacity of HUVECs. By using the angiogenesis antibody array, we found that these activities were associated with a reduction in expression levels of the pro-angiogenic factors, *MMP9* and *VEGFA*, which were consistently reduced in *ADAMTS9* transfectants. Based on the deletion patterns of the *ADAMTS9* transcripts in tumors and a TS derived from the tumorigenic transfectants, we speculate that the tumor-suppressive activity of *ADAMTS9* in ESCC was associated with the thrombospondin (TSP) domains in the C-terminal region of the gene. Taken together, our data strongly suggest that *ADAMTS9* plays a critical role in the "angiogenic switch" and transforms in the ESCC cell lines from a pro-angiogenic to a non-angiogenic phenotype.

4.2 Chromosome 9: *ENG, DEC1*

The transfer of chromosome 9 containing an interstitial deletion at 9p21 (where the well-known TSG, *p16* is located) to NPC HONE1 cell line did not result in tumor suppression in the nude mouse assay, but *p16* cDNA suppressed growth of HONE1 cells *in vitro* assay. It suggests that *p16* gene plays an important role in this cancer (Cheng et al., 2000). The similar transfer of chromosome 9 into SLMT1 provided the first functional tumor suppressive evidence in ESCC (Yang et al 2005). The result suggested that gene(s) other than p16 on chromosome 9 is (are) important for ESCC tumorigenesis. The ESCC chromosome 9 MCHs exhibited a delayed latency period in tumor formation compared with that of the parental SLMT1 cells. The delay in tumor growth kinetics was hypothesized to be associated with the loss or inactivation of wild type alleles from the exogenous transferred donor chromosome 9. Detailed microsatellite marker-PCR deletion mapping analysis of the tumor suppressive

chromosome 9 MCHs and their corresponding derived TSs delineated that the critical regions that may harbor candidate TSGs to a 2.4 Mb region at 9q33-q34 around D9S112.

4.2.1 *ENG (Endoglin)*

The MMCT-identified CR at 9q32-34 is a gene-rich region. *ENG (Endoglin*), mapping to 9q33-q34.1, is a component of the transforming growth factor beta (TGF-β) receptor complex and is involved in tumor angiogenesis by modulating the biological effect of TGF-β. Significant down-regulation of *ENG* was detected at frequencies of 87.5% in 16 ESCC cell lines, 39.1% directly in 23 ESCC tumor specimens from Hong Kong, and 33.4% in 18 ESCC tumor specimens from the high-risk ESCC region of Henan, China. Both epigenetic methylation and allelic loss appear to contribute to *ENG* down-regulation in ESCC tumors. Subsequent functional studies with restoration of *ENG* in an ESCC cell line demonstrated that *ENG* plays a critical role in ESCC carcinogenesis. Colony formation efficiency was significantly reduced by over-expression of *ENG*. In addition, significantly smaller colonies of *ENG* stable transfectants were formed in Matrigel culture. Significant suppression of invasion efficiency and tumorigenicity were also observed, when comparing the *ENG* stable transfectants with the vector-alone transfectants. No report had yet verified the functional role of *ENG* in ESCC tumor cells. This study provides evidence supporting *ENG*, as a cell invasion and tumor-suppressing gene in ESCC. *ENG* may be functionally involved in TGF-β signaling. Down-regulation of *ENG* in esophageal cancer in this study may provoke cancer progression through blocking the tumor suppression of the TGF-β signaling cascade. For the functional impact of *ENG* in cell migration, *ENG* may suppress cancer cell motility by a TGF-β-dependent mechanism involving activation of the type I TGF-β receptor and Smad1, as reported in the study of prostate cancer cells (Craft et al 2007). In endothelial cells, high endoglin expression stimulates the type I activin receptor-like kinases (ALK1) pathway and indirectly inhibits ALK5 signaling for endothelial cell proliferation and migration, thus promoting the state of angiogenesis. *ENG* may act differently in cancer cell versus endothelial cells. *ENG* may suppress cancer cell proliferation in the pre-malignant stage; meanwhile, its expression could promote angiogenesis facilitating the cancer progression in the late malignant stage. The mechanism of ENG in suppressing ECSC tumor requires further study (Wong et al 2008).

4.2.2 *DEC1 (Deleted in Esophageal Cancer 1)*

DEC1 (*Deleted in Esophageal Cancer 1*) is in the vicinity of the CR at 9q32-34 and is down-regulated frequently in ESCC cell lines and tumor tissues (Leung et al 2008). The DEC1 protein localizes to both the cytoplasm and nucleus. The vesicular pattern of DEC1 in the cytoplasm appears to localize at the Golgi and Golgi-endoplasmic reticulum intermediate compartment. DEC1 is clinically important as the tissue microarray (TMA) study suggested an association of DEC1 expression with lymph node metastasis, early onset ESCC, and familial status (Wong et al 2011b). DEC1 stably transfected clones provided functional evidence for cell growth inhibition in vitro and significant delay in tumor growth in vivo (Yang et al., 2005). DEC1 stable clones showed significantly fewer colony numbers as compared to the vector-alone control. Restoration of DEC1 expression also negatively affected anchorage-independent growth properties of an ESCC cell line (Leung et al 2008). Using cDNA microarray analysis to reveal the differential expression profiling between tumor suppressive *DEC1* clones versus the vector-alone transfectant, *DUSP6* (*dual-specificity*

phosphatase 6) was identified as one of the downstream targets of *DEC1*, as it is up-regulated in *DEC1* stable transfectants, C4 and C9, compared to vector-alone stable transfectants. It is expected that in clinical specimens, the higher expression of *DEC1* associates with higher expression of *DUSP6*. This association was observed in tumor tissues in younger aged ESCC patients group. This association was only significant in these two groups, which was limited by the number of available samples (only 26 normal counterpart tissues and 74 tumor tissues). Subsequent functional study of *DUSP* also indicated that *DUSP6* plays a crucial role for ESCC carcinogenesis by inhibiting cell invasion and impairing the epithelial-mesenchymal transition (EMT)-associated phenotype (Wong et al 2011a).

4.3 Chromosome 13: *THSD1*

Chromosome 13q deletions are frequent events in several human cancers, including ESCC (Hu et al 2000, Pack et al 1999), nasopharyngeal (Tsang et al 1999) and lung (Tamura et al 1997) cancers. Molecular evidence of gross deletions which implicate the existence of TSGs came from numerous LOH and CGH studies. Our group has pioneered the identification of the TSGs on chromosome 13 by the functional complementation approach in both NPC and ESCC (Cheng et al 2004, Ko et al 2008). The transfer of intact chromosome 13 in HONE1 cells identified a critical region essential for the viability and growth of NPC MCHs at chromosome 13q12, but it was not RB gene (Cheng et al 2004). By the microsatellite deletion mapping, D13S893 at 13q12, a minimally deleted region of 0.7 Mb, was found to be non-randomly eliminated in the six chromosome 13 MCHs bounded by markers D13S1287 and D13S260. The growth suppressive activity involved at least one novel growth control gene for NPC tumorigenesis. Our subsequent study employed the same MMCT technique in an ESCC model with the ESCC cell line, SLMT1. The transfer of an intact chromosome 13 into this highly tumorigenic recipient cell line conferred tumor suppressive activity, and identified critical regions at 13q12.3, 13q14.11, and 13q14.3. Of interest, a 0.373 Mb at the critical region 2 (CR2), mapped to 13q12.3 and was co-localized to the same CR identified in the NPC model system in our chromosome 13 transfer study (Cheng et al 2004, Ko et al 2008).

4.3.1 *THSD1 (Thrombospondin type I domain-containing 1)*

The ESCC functional studies implicate the importance of chromosome 13q14 in tumor suppression; TS microsatellite-deletion mapping analysis localized two CRs (CR3 at D13S263 and CR4 at D13S133) at chromosomal region 13q14, which are frequently eliminated (Ko et al 2008). Differential gene expression profiles of a reference immortalized normal esophageal epithelial cell line, three tumor-suppressing MCHs, and their tumorigenic parental SLMT1 cell line were revealed by cDNA oligonucleotide microarray analysis. Nine 13q14 candidates genes, including *RB1*, were identified to show down-regulation in SLMT-1 as compared to NE1, the immortalized normal esophageal epithelial cell line, and the MCHs. *RB1* is a well-known TSG mapped to 13q14, but our Western blot analysis indicated that the active form of RB was not increased in the tumor suppressive MCHs (data not shown). The data suggested that novel candidate TSG(s) other than *RB1* should be involved in the observed tumor suppression. RT-PCR was performed for *KIAA0853*, *ESD*, *CHC1L*, *PHF11*, *RFP2*, *FLJ11712*, *THSD1*, and *C13orf9*. Real-time PCR results validated the frequent down-regulation of *THSD1* and *PHF11* in ESCC cell lines. *THSD1* is located between *FLJ11712* and *C13orf9* within 13q14.3, but only specific loss of *THSD1* expression in all cancer cell lines was detected. Since *THSD1* showed a more prominent loss

than that of *PHF11*, it was the first target chosen for further functional characterization. Epigenetic silencing and LOH were the mechanisms responsible, at least in part, for the loss of *THSD1* expression in ESCC tumorigenesis. The wild type *THSD1* transfection in SLMT1 resulted in significant reduction of colony formation ability, providing evidence for a growth suppressive role of *THSD1* in ESCC tumorigenesis.
The function of *THSD1* is unknown. It encodes a transmembrane molecule containing a thrombospondin type 1 repeat (TSR), which may be involved in cell adhesion and angiogenesis (de Fraipont et al 2001). Interestingly, analysis of the differential expression levels of this gene in previous microarray studies show that high *THSD1* expression positively correlated with a better distant metastasis survival in breast cancer patients. This is consistent with its loss possibly being associated with metastatic tumor spread; studies are needed to evaluate its potential importance as a biomarker for esophageal carcinoma. Further functional studies on *THSD1* are now underway to elucidate its tumor suppressive role.

4.4 Chromosome 14: *LTBP-2*

Chromosome 14 loss is commonly found in different cancers, including esophageal (Ihara et al 2002), renal (Yoshimoto et al 2007), lung (Weir et al 2007), and colon cancers (Mourra et al 2007). In NPC extensive chromosome 14 allelic loss has been reported (Dodd et al 2006, Lo et al 2000, Lung et al 2001, Shao et al 2002). This suggests the importance of chromosome 14 in tumor development. In our earlier NPC study, chromosome 14q11.2-13.1 and 14q32.1 regions were found to associate with tumor suppression. In those chromosome 14 MCHs, non-random eliminations of two CRs were consistently observed and associated with tumor growth in tumorigenicity assays (Cheng et al 2003). In a later study, a new panel of chromosome 14 MCHs with an intact exogenous chromosome 14 was established. The potent ability to suppress tumor growth in the *in vivo* tumorigenicity assay of all intact chromosome 14 MCHs suggests the ability of chromosome 14 to suppress tumor formation in HONE1 cells. This is consistent with chromosome 14 harboring candidate TSGs involved in NPC development (Cheung et al 2009).
An intact chromosome 14 was also transferred into the ESCC SLMT1 cell line. The tumorigenic potential of microcell hybrids containing the transferred chromosome 14 provided functional evidence that tumor-suppressive regions of chromosome 14 are essential for esophageal cancer. TSs emerging in nude mice during the tumorigenicity assay was analyzed by detailed PCR-microsatellite typing and dual-colour BAC FISH to identify critical non-randomly eliminated regions. A 680-kb CR mapped to 14q32.13 and an approximately 2.2-Mb CR mapped to 14q32.33 were delineated. (Ko et al 2005). Microarray differential gene expression profiling of tumor-suppressive chromosome 14 MCH cell lines and their tumorigenic TSs identified *LTBP-2* (*latent transforming growth factor β binding protein 2*) mapped to 14q24 as a candidate TSG important for ESCC (Chan et al 2011).

4.4.1 *LTBP-2 (Latent transforming growth factor β binding protein 2)*

The extracellular matrix (ECM) protein *LTBP-2* (*Latent transforming growth factor β binding protein 2)* encodes a secretary protein that functions as a component of the ECM microfibrils and belongs to the LTBP/fibrillin family (Chan et al 2011). Unlike other members in LTBP

family, LTBP-2 does not form complexes with the small latent TGF-βs. Interestingly, in addition to the first reported tumor suppressor role in ESCC, *LTBP-2* is related to congenital glaucoma and rheumatoid arthritis which are eye and bone diseases, respectively. LTBP-2 expression at the mRNA and protein levels was down-regulated in both ESCC cell lines and primary tumors. One of the mechanisms responsible for the down-regulation of *LTBP-2* is via promoter hypermethylation. Restoration of *LTBP-2* in an ESCC cancer cell line resulted in tumor suppression in nude mouse assay, which is partially explained by the significant reduction of colony-forming ability on matrigel 3D culture and anchorage-independent growth *in vitro*. Further *in vitro* functional characterization of *LTBP-2* demonstrated its inhibitory role in angiogenesis, migration, and invasion of cancer cells. An angiogenesis protein array analysis of conditioned medium from *LTBP-2* stable clone revealed the change in expressions of different cytokines, including GM-CSF, RANTES, VEGF, uPAR, I-309, MMP-1, Angiopoietin-1, and MCP-1, which in turn induce a less favorable microenvironment for angiogenesis and tumor growth. ESCC is a deadly disease and patients are usually diagnosed at late stage. In many late stage tumors, the TGF-β signaling pathway is involved in the activation of EMT program, which is responsible for cancer cell traits promoting malignancy. In contrast, TGF-β is well-known to be anti-proliferative. Interestingly, an inverse correlation of high LTBP-2 and survival in advanced ESCC stage was detected by IHC staining of primary ESCC tissues. LTBP-2 may indirectly regulate TGF-β by competing with LTBP-1 for fibrillin-1 binding site (Hirani et al 2007).

5. Conclusions

In this book chapter, we focus on using MMCT, as a functional approach to identify candidate TSGs. The monochromosome transfers of selected chromosomes into the NPC and ESCC cell lines, HONE1 and SLMT1, were performed to determine whether tumor suppressing activities for NPC and ESCC mapped to chromosomes 3, 9, 11, 13, 14 and 17, as described in our previous reports. While all these experiments have been performed in the two NPC and SLMT1 cell lines HONE1 and SLMT1, it should be appreciated there are a few of well-established and well-characterized NPC and ESCC cell lines available for this kind of study. Not surprisingly, identification of TSGs or regions in these cell lines was subsequently confirmed to be important in other NPC and ESCC cell lines as well as primary tumors. By using the MMCT approach, we successfully identified several CRs associated with tumor suppression in the HONE1/SLMT1 cell line systems. These candidate TSGs mapping to these regions were subsequently studied for their role in tumor suppression assays. We discovered that *ADAMTS9* at 3p14.2, *ENG* and *DEC1* at 9q33-q34, are genes mapped into CRs, and important for tumor suppression in ESCC. Gene expression profiling of the 19K and 28K oligonucleotide microarrays, including tumor-suppressive MCH and tumorigenic TS cell lines, was utilized to identify candidate genes (*THSD1* and *LTBP-2*) within the critical tumor suppressive regions. It is clear now that many genes contribute to the development of these two important cancers.

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7. References

Anderson MJ and Stanbridge EJ (1993). Tumor suppressor genes studied by cell hybridization and chromosome transfer. *Faseb J* 7: 826-833.

Apte SS (2004). A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motifs: the ADAMTS family. *Int J Biochem Cell Biol* 36: 981-985.

Chan SH, Yee Ko JM, Chan KW, Chan YP, Tao Q, Hyytiainen M, Keski-Oja J, Law S, Srivastava G, Tang J, Tsao SW, Chen H, Stanbridge EJ and Lung ML (2011). The ECM protein LTBP-2 is a suppressor of esophageal squamous cell carcinoma tumor formation but higher tumor expression associates with poor patient outcome. *Int J Cancer* 129: 565-573.

Cheng Y, Poulos NE, Lung ML, Hampton G, Ou B, Lerman MI and Stanbridge EJ (1998). Functional evidence for a nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. *Proc Natl Acad Sci U S A* 95: 3042-3047.

Cheng Y, Stanbridge EJ, Kong H, Bengtsson U, Lerman MI and Lung ML (2000). A functional investigation of tumor suppressor gene activities in a nasopharyngeal carcinoma cell line HONE1 using a monochromosome transfer approach. *Genes Chromosomes Cancer* 28: 82-91.

Cheng Y, Chakrabarti R, Garcia-Barcelo M, Ha TJ, Srivatsan ES, Stanbridge EJ and Lung ML (2002). Mapping of nasopharyngeal carcinoma tumor-suppressive activity to a 1.8-megabase region of chromosome band 11q13. *Genes Chromosomes Cancer* 34: 97-103.

Cheng Y, Ko JM, Lung HL, Lo PH, Stanbridge EJ and Lung ML (2003). Monochromosome transfer provides functional evidence for growth-suppressive genes on chromosome 14 in nasopharyngeal carcinoma. *Genes Chromosomes Cancer* 37: 359-368.

Cheng Y, Lung HL, Wong PS, Hao DC, Man CS, Stanbridge EJ and Lung ML (2004). Chromosome 13q12 region critical for the viability and growth of nasopharyngeal carcinoma hybrids. *Int J Cancer* 109: 357-362.

Cheung AK, Lung HL, Ko JM, Cheng Y, Stanbridge EJ, Zabarovsky ER, Nicholls JM, Chua D, Tsao SW, Guan XY and Lung ML (2009). Chromosome 14 transfer and functional studies identify a candidate tumor suppressor gene, mirror image polydactyly 1, in nasopharyngeal carcinoma. *Proc Natl Acad Sci U S A* 106: 14478-14483.

Clark ME, Kelner GS, Turbeville LA, Boyer A, Arden KC and Maki RA (2000). ADAMTS9, a novel member of the ADAM-TS/ metallospondin gene family. *Genomics* 67: 343-350.

Craft CS, Romero D, Vary CP and Bergan RC (2007). Endoglin inhibits prostate cancer motility via activation of the ALK2-Smad1 pathway. *Oncogene* 26: 7240-7250.

Daly JM, Fry WA, Little AG, Winchester DP, McKee RF, Stewart AK and Fremgen AM (2000). Esophageal cancer: results of an American College of Surgeons Patient Care Evaluation Study. *J Am Coll Surg* 190: 562-572; discussion 572-563.

de Fraipont F, Nicholson AC, Feige JJ and Van Meir EG (2001). Thrombospondins and tumor angiogenesis. *Trends Mol Med* 7: 401-407.

Dodd LE, Sengupta S, Chen IH, den Boon JA, Cheng YJ, Westra W, Newton MA, Mittl BF, McShane L, Chen CJ, Ahlquist P and Hildesheim A (2006). Genes involved in DNA repair and nitrosamine metabolism and those located on chromosome 14q32 are dysregulated in nasopharyngeal carcinoma. *Cancer Epidemiol Biomarkers Prev* 15: 2216-2225.

Dowdy SF, Scanlon DJ, Fasching CL, Casey G and Stanbridge EJ (1990). Irradiation microcell-mediated chromosome transfer (XMMCT): the generation of specific chromosomal arm deletions. *Genes Chromosomes Cancer* 2: 318-327.

Dowdy SF, Fasching CL, Araujo D, Lai KM, Livanos E, Weissman BE and Stanbridge EJ (1991). Suppression of tumorigenicity in Wilms tumor by the p15.5-p14 region of chromosome 11. *Science* 254: 293-295.

Fournier RE and Ruddle FH (1977a). Stable association of the human transgenome and host murine chromosomes demonstrated with trispecific microcell hybrids. *Proc Natl Acad Sci U S A* 74: 3937-3941.

Fournier RE and Ruddle FH (1977b). Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. *Proc Natl Acad Sci U S A* 74: 319-323.

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM and Dryja TP (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 323: 643-646.

Goyette MC, Cho K, Fasching CL, Levy DB, Kinzler KW, Paraskeva C, Vogelstein B and Stanbridge EJ (1992). Progression of colorectal cancer is associated with multiple tumor suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect via chromosome transfer. *Mol Cell Biol* 12: 1387-1395.

Hanahan D and Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell* 144: 646-674.

Harris H, Miller OJ, Klein G, Worst P and Tachibana T (1969). Suppression of malignancy by cell fusion. *Nature* 223: 363-368.

Hesson LB, Cooper WN and Latif F (2007). Evaluation of the 3p21.3 tumor-suppressor gene cluster. *Oncogene* 26:7283-7301

Hirani R, Hanssen E and Gibson MA (2007). LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein. *Matrix Biol* 26: 213-223.

Holmes RS and Vaughan TL (2007). Epidemiology and pathogenesis of esophageal cancer. *Semin Radiat Oncol* 17: 2-9.

Hu N, Roth MJ, Polymeropolous M, Tang ZZ, Emmert-Buck MR, Wang QH, Goldstein AM, Feng SS, Dawsey SM, Ding T, Zhuang ZP, Han XY, Ried T, Giffen C and Taylor PR (2000). Identification of novel regions of allelic loss from a genomewide scan of esophageal squamous-cell carcinoma in a high-risk Chinese population. *Genes Chromosomes Cancer* 27: 217-228.

Huang DP, Ho JH, Chan WK, Lau WH and Lui M (1989). Cytogenetics of undifferentiated nasopharyngeal carcinoma xenografts from southern Chinese. *Int J Cancer* 43: 936-939.

Ihara Y, Kato Y, Bando T, Yamagishi F, Minamimura T, Sakamoto T, Tsukada K and Isobe M (2002). Allelic imbalance of 14q32 in esophageal carcinoma. *Cancer Genet Cytogenet* 135: 177-181.

Jeyakumar A, Brickman TM and Doerr T (2006). Review of nasopharyngeal carcinoma. *Ear Nose Throat J* 85: 168-170, 172-163, 184.

Jin H, Wang X, Ying J, Wong AH, Li H, Lee KY, Srivastava G, Chan AT, Yeo W, Ma BB, Putti TC, Lung ML, Shen ZY, Xu LY, Langford C and Tao Q (2007). Epigenetic identification of ADAMTS18 as a novel 16q23.1 tumor suppressor frequently silenced in esophageal, nasopharyngeal and multiple other carcinomas. *Oncogene* 26: 7490-7498.

Ko JM, Yau WL, Chan PL, Lung HL, Yang L, Lo PH, Tang JC, Srivastava G, Stanbridge EJ and Lung ML (2005). Functional evidence of decreased tumorigenicity associated with monochromosome transfer of chromosome 14 in esophageal cancer and the mapping of tumor-suppressive regions to 14q32. *Genes Chromosomes Cancer* 43: 284-293.

Ko JM, Chan PL, Yau WL, Chan HK, Chan KC, Yu ZY, Kwong FM, Miller LD, Liu ET, Yang LC, Lo PH, Stanbridge EJ, Tang JC, Srivastava G, Tsao SW, Law S and Lung ML (2008). Monochromosome transfer and microarray analysis identify a critical tumor-suppressive region mapping to chromosome 13q14 and THSD1 in esophageal carcinoma. *Mol Cancer Res* 6: 592-603.

Koi M, Morita H, Yamada H, Satoh H, Barrett JC and Oshimura M (1989). Normal human chromosome 11 suppresses tumorigenicity of human cervical tumor cell line SiHa. *Mol Carcinog* 2: 12-21.

Koi M, Johnson LA, Kalikin LM, Little PF, Nakamura Y and Feinberg AP (1993). Tumor cell growth arrest caused by subchromosomal transferable DNA fragments from chromosome 11. *Science* 260: 361-364.

Lerman MI, Minna JD (2000). The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Cancer Res.* 60:6116-6133.

Leung AC, Wong VC, Yang LC, Chan PL, Daigo Y, Nakamura Y, Qi RZ, Miller LD, Liu ET, Wang LD, Li JL, Law S, Tsao SW and Lung ML (2008). Frequent decreased expression of candidate tumor suppressor gene, DEC1, and its anchorage-independent growth properties and impact on global gene expression in esophageal carcinoma. *Int J Cancer* 122: 587-594.

Li LC and Dahiya R (2002). MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 18: 1427-1431.

Lin CY, Strom A, Vega VB, Kong SL, Yeo AL, Thomsen JS, Chan WC, Doray B, Bangarusamy DK, Ramasamy A, Vergara LA, Tang S, Chong A, Bajic VB, Miller LD, Gustafsson JA and Liu ET (2004). Discovery of estrogen receptor alpha target genes and response elements in breast tumor cells. *Genome Biol* 5: R66.

Lo KW, Teo PM, Hui AB, To KF, Tsang YS, Chan SY, Mak KF, Lee JC and Huang DP (2000). High resolution allelotype of microdissected primary nasopharyngeal carcinoma. *Cancer Res* 60: 3348-3353.

Lo KW, Kwong J, Hui AB, Chan SY, To KF, Chan AS, Chow LS, Teo PM, Johnson PJ, Huang DP (2001). High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res. 61:3877-3881.

Lo PH, Leung AC, Kwok CY, Cheung WS, Ko JM, Yang LC, Law S, Wang LD, Li J, Stanbridge EJ, Srivastava G, Tang JC, Tsao SW and Lung ML (2007). Identification of a tumor suppressive critical region mapping to 3p14.2 in esophageal squamous cell carcinoma and studies of a candidate tumor suppressor gene, ADAMTS9. *Oncogene* 26: 148-157.

Lo PH, Lung HL, Cheung AK, Apte SS, Chan KW, Kwong FM, Ko JM, Cheng Y, Law S, Srivastava G, Zabarovsky ER, Tsao SW, Tang JC, Stanbridge EJ and Lung ML (2010). Extracellular protease ADAMTS9 suppresses esophageal and nasopharyngeal carcinoma tumor formation by inhibiting angiogenesis. *Cancer Res* 70: 5567-5576.

Lung HL, Lo CC, Wong CC, Cheung AK, Cheong KF, Wong N, Kwong FM, Chan KC, Law EW, Tsao SW, Chua D, Sham JS, Cheng Y, Stanbridge EJ, Robertson GP and Lung ML (2008a). Identification of tumor suppressive activity by irradiation microcell-mediated chromosome transfer and involvement of alpha B-crystallin in nasopharyngeal carcinoma. *Int J Cancer* 122: 1288-1296.

Lung HL, Lo PH, Xie D, Apte SS, Cheung AK, Cheng Y, Law EW, Chua D, Zeng YX, Tsao SW, Stanbridge EJ and Lung ML (2008b). Characterization of a novel epigenetically-silenced, growth-suppressive gene, ADAMTS9, and its association with lymph node metastases in nasopharyngeal carcinoma. *Int J Cancer* 123: 401-408.

Lung ML, Choi CV, Kong H, Yuen PW, Kwong D, Sham J and Wei WI (2001). Microsatellite allelotyping of chinese nasopharyngeal carcinomas. *Anticancer Res* 21: 3081-3084.

Mitelman F, Mark-Vendel E, Mineur A, Giovanella B and Klein G (1983). A 3q+ marker chromosome in EBV-carrying nasopharyngeal carcinomas. *Int J Cancer* 32: 651-655.

Mourra N, Zeitoun G, Buecher B, Finetti P, Lagarde A, Adelaide J, Birnbaum D, Thomas G and Olschwang S (2007). High frequency of chromosome 14 deletion in early-onset colon cancer. *Dis Colon Rectum* 50: 1881-1886.

Murakami Y, Nobukuni T, Tamura K, Maruyama T, Sekiya T, Arai Y, Gomyou H, Tanigami A, Ohki M, Cabin D, Frischmeyer P, Hunt P and Reeves RH (1998a). Localization of tumor suppressor activity important in nonsmall cell lung carcinoma on chromosome 11q. *Proc Natl Acad Sci U S A* 95: 8153-8158.

Murakami Y and Sekiya T (1998b). Accumulation of genetic alterations and their significance in each primary human cancer and cell line. *Mutat Res* 400: 421-437.

Murakami Y (2002). Functional cloning of a tumor suppressor gene, TSLC1, in human non-small cell lung cancer. *Oncogene* 21: 6936-6948.

Murakami YS, Brothman AR, Leach RJ and White RL (1995). Suppression of malignant phenotype in a human prostate cancer cell line by fragments of normal chromosomal region 17q. *Cancer Res* 55: 3389-3394.

Murayama F and Okada Y (1965). Effect of calcium on the cell fusion reaction caused by HVJ. *Biken J* 8: 103-105.

Pachnis V, Pevny L, Rothstein R and Costantini F (1990). Transfer of a yeast artificial chromosome carrying human DNA from Saccharomyces cerevisiae into mammalian cells. *Proc Natl Acad Sci U S A* 87: 5109-5113.

Pack SD, Karkera JD, Zhuang Z, Pak ED, Balan KV, Hwu P, Park WS, Pham T, Ault DO, Glaser M, Liotta L, Detera-Wadleigh SD and Wadleigh RG (1999). Molecular cytogenetic fingerprinting of esophageal squamous cell carcinoma by comparative genomic hybridization reveals a consistent pattern of chromosomal alterations. *Genes Chromosomes Cancer* 25: 160-168.

Pavan WJ, Hieter P and Reeves RH (1990a). Generation of deletion derivatives by targeted transformation of human-derived yeast artificial chromosomes. *Proc Natl Acad Sci U S A* 87: 1300-1304.

Pavan WJ, Hieter P and Reeves RH (1990b). Modification and transfer into an embryonal carcinoma cell line of a 360-kilobase human-derived yeast artificial chromosome. *Mol Cell Biol* 10: 4163-4169.

Porter S, Clark IM, Kevorkian L and Edwards DR (2005). The ADAMTS metalloproteinases. *Biochem J* 386: 15-27.

Qi Y, Chiu JF, Wang L, Kwong DL and He QY (2005). Comparative proteomic analysis of esophageal squamous cell carcinoma. *Proteomics* 5: 2960-2971.

Robertson GP, Hufford A and Lugo TG (1997). A panel of transferable fragments of human chromosome 11q. *Cytogenet Cell Genet* 79: 53-59.

Saxon PJ, Srivatsan ES, Leipzig GV, Sameshima JH and Stanbridge EJ (1985). Selective transfer of individual human chromosomes to recipient cells. *Mol Cell Biol* 5: 140-146.

Saxon PJ, Srivatsan ES and Stanbridge EJ (1986). Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells. *EMBO J* 5: 3461-3466.

Shao J, Li Y, Wu Q, Liang X, Yu X, Huang L, Hou J, Huang X, Ernberg I, Hu LF and Zeng Y (2002). High frequency loss of heterozygosity on the long arms of chromosomes 13 and 14 in nasopharyngeal carcinoma in Southern China. *Chin Med J (Engl)* 115: 571-575.

Stanbridge EJ (1976). Suppression of malignancy in human cells. *Nature* 260: 17-20.

Stanbridge EJ (1992). Functional evidence for human tumour suppressor genes: chromosome and molecular genetic studies. *Cancer Surv* 12: 5-24.

Tamura K, Zhang X, Murakami Y, Hirohashi S, Xu HJ, Hu SX, Benedict WF and Sekiya T (1997). Deletion of three distinct regions on chromosome 13q in human non-small-cell lung cancer. *Int J Cancer* 74: 45-49.

Todd MC, Xiang RH, Garcia DK, Kerbacher KE, Moore SL, Hensel CH, Liu P, Siciliano MJ, Kok K, van den Berg A, Veldhuis P, Buys CH, Killary AM and Naylor SL (1996). An 80 Kb P1 clone from chromosome 3p21.3 suppresses tumor growth in vivo. *Oncogene* 13: 2387-2396.

Tsang YS, Lo KW, Leung SF, Choi PH, Fong Y, Lee JC and Huang DP (1999). Two distinct regions of deletion on chromosome 13q in primary nasopharyngeal carcinoma. *Int J Cancer* 83: 305-308.

Vazquez F, Hastings G, Ortega MA, Lane TF, Oikemus S, Lombardo M and Iruela-Arispe ML (1999). METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity. *J Biol Chem* 274: 23349-23357.

Wada M, Ihara Y, Tatsuka M, Mitsui H, Kohno K, Kuwano M and Schlessinger D (1994). HPRT yeast artificial chromosome transfer into human cells by four methods and an involvement of homologous recombination. *Biochem Biophys Res Commun* 200: 1693-1700.

Wei WI and Sham JS (2005). Nasopharyngeal carcinoma. *Lancet* 365: 2041-2054.

Weir BA, Woo MS, Getz G, Perner S, Ding L, Beroukhim R, Lin WM, Province MA, Kraja A, Johnson LA, Shah K, Sato M, Thomas RK, Barletta JA, Borecki IB, Broderick S, Chang AC, Chiang DY, Chirieac LR, Cho J, Fujii Y, Gazdar AF, Giordano T, Greulich H, Hanna M, Johnson BE, Kris MG, Lash A, Lin L, Lindeman N, Mardis ER, McPherson JD, Minna JD, Morgan MB, Nadel M, Orringer MB, Osborne JR, Ozenberger B, Ramos AH, Robinson J, Roth JA, Rusch V, Sasaki H, Shepherd F, Sougnez C, Spitz MR, Tsao MS, Twomey D, Verhaak RG, Weinstock GM, Wheeler DA, Winckler W, Yoshizawa A, Yu S, Zakowski MF, Zhang Q, Beer DG, Wistuba, II, Watson MA, Garraway LA, Ladanyi M, Travis WD, Pao W, Rubin MA, Gabriel SB, Gibbs RA, Varmus HE, Wilson RK, Lander ES and Meyerson M (2007). Characterizing the cancer genome in lung adenocarcinoma. *Nature* 450: 893-898.

Weissman BE, Saxon PJ, Pasquale SR, Jones GR, Geiser AG and Stanbridge EJ (1987). Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression. *Science* 236: 175-180.

Wong VC, Chan PL, Bernabeu C, Law S, Wang LD, Li JL, Tsao SW, Srivastava G and Lung ML (2008). Identification of an invasion and tumor-suppressing gene, Endoglin (ENG), silenced by both epigenetic inactivation and allelic loss in esophageal squamous cell carcinoma. *Int J Cancer* 123: 2816-2823.

Wong VC, Chen H, Ko JM, Chan KW, Chan YP, Law S, Chua D, Kwong DL, Lung HL, Srivastava G, Tang JC, Tsao SW, Zabarovsky ER, Stanbridge EJ and Lung ML (2011a). Tumor suppressor dual-specificity phosphatase 6 (DUSP6) impairs cell invasion and epithelial-mesenchymal transition (EMT)-associated phenotype. *Int J Cancer*.

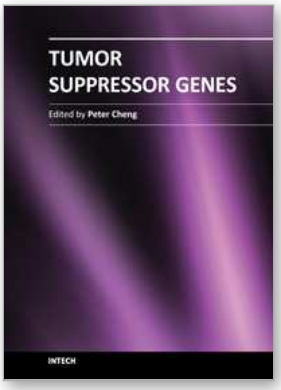
Wong VC, Ko JM, Qi RZ, Li PJ, Wang LD, Li JL, Chan YP, Chan KW, Stanbridge EJ and Lung ML (2011b). Abrogated expression of DEC1 during oesophageal squamous cell carcinoma progression is age- and family history-related and significantly associated with lymph node metastasis. *Br J Cancer* 104: 841-849.

Wu X, Chen VW, Ruiz B, Andrews P, Su LJ and Correa P (2006). Incidence of esophageal and gastric carcinomas among American Asians/Pacific Islanders, whites, and blacks: subsite and histology differences. *Cancer* 106: 683-692.

Yang L, Leung AC, Ko JM, Lo PH, Tang JC, Srivastava G, Oshimura M, Stanbridge EJ, Daigo Y, Nakamura Y, Tang CM, Lau KW, Law S and Lung ML (2005). Tumor suppressive role of a 2.4 Mb 9q33-q34 critical region and DEC1 in esophageal squamous cell carcinoma. *Oncogene* 24: 697-705.

Yau WL, Lung HL, Zabarovsky ER, Lerman MI, Sham JS, Chua DT, Tsao SW, Stanbridge EJ, Lung ML (2006). Functional studies of the chromosome 3p21.3 candidate tumor suppressor gene BLU/ZMYND10 in nasopharyngeal carcinoma. *Int J Cancer* 119:2821-2826.

Yoshimoto T, Matsuura K, Karnan S, Tagawa H, Nakada C, Tanigawa M, Tsukamoto Y, Uchida T, Kashima K, Akizuki S, Takeuchi I, Sato F, Mimata H, Seto M and Moriyama M (2007). High-resolution analysis of DNA copy number alterations and gene expression in renal clear cell carcinoma. *J Pathol* 213: 392-401.



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Functional evidence obtained from somatic cell fusion studies indicated that a group of genes from normal cells might replace or correct a defective function of cancer cells. Tumorigenesis that could be initiated by two mutations was established by the analysis of hereditary retinoblastoma, which led to the eventual cloning of RB1 gene. The two-hit hypothesis helped isolate many tumor suppressor genes (TSG) since then. More recently, the roles of haploinsufficiency, epigenetic control, and gene dosage effects in some TSGs, such as P53, P16 and PTEN, have been studied extensively. It is now widely recognized that deregulation of growth control is one of the major hallmarks of cancer biological capabilities, and TSGs play critical roles in many cellular activities through signaling transduction networks. This book is an excellent review of current understanding of TSGs, and indicates that the accumulated TSG knowledge has opened a new frontier for cancer therapies.

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