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MicroRNA-related genetic variations as predictors for risk of second primary tumor and/or recurrence in patients with early-stage head and neck cancer

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Second primary tumor (SPT) and/or recurrence negatively impact the prognosis of patients with curatively treated early-stage head and neck cancer. MicroRNAs (miRNAs) play important roles in cancer development. We explored whether the variations of miRNA-related pathway were associated with the risk of SPT/recurrence in patients with early-stage head and neck cancer. This study includes 150 early-stage head and neck cancer patients with SPT/recurrence and 300 patients without SPT/recurrence. Two hundred and thirty-five tagging and potentially functional single-nucleotide polymorphisms (SNPs) were genotyped from eight miRNA biogenesis pathway genes and 135 miRNA-targeted genes. Eighteen miRNA-related SNPs were significantly associated with the risk of SPT/recurrence. The most significant SNP was rs3747238, a miRNA-binding site SNP in *SMC1B*. The variant homozygous genotype of this SNP was associated with a 1.74-fold increased risk [95% confidence interval (CI) 1.19–2.54; $P = 0.004$]. Cumulative effect analysis showed joint effects for the number of unfavorable genotype in patients. Survival tree analysis further identified the high-order gene–gene interactions and categorized the study subjects into low-, medium- and high-risk groups. Patients in the high-risk group had a 4.84-fold increased risk (95% CI: 3.11–7.51; $P = 2.45 \times 10^{-12}$) and a shorter event-free median survival time of 37.9 months (log rank $P = 2.28 \times 10^{-13}$). Our results suggested that miRNA-related genetic polymorphisms may be used individually and jointly to predict the risk of SPT/recurrence of early-stage head and neck cancer patients.

Introduction

Head and neck cancers account for 3–5% of all cancers in the USA (1,2). It was estimated that >48 000 (35 720 oral and pharynx + 12 290 larynx) Americans developed head and neck cancer in 2009 and nearly 11 260 (7600 oral and pharynx + 3660 larynx) died from this disease (3). Most early-stage head and neck cancer patients can be cured with surgery, radiotherapy and chemotherapy. However, second primary tumors (SPTs) and local–regional recurrence negatively impact their long-term prognosis. It has been reported that ∼15 to 25% of head and neck cancer patients will develop SPT/recurrence during the first 5 years after initial diagnosis (4–8). Thus, to develop and identify clinically applicable biomarkers to predict SPT/recurrence is important for the surveillance and targeted chemoprevention of high-risk patients. Genetic variations within various oncogenic pathways such as cell cycle progression, DNA repair and immune modulation have been associated with the etiology and prognosis of head and neck cancers (9,10). In this study, we sought to evaluate the associations of the genetic variations in the microRNA (miRNA) biogenesis pathway genes and miRNA-binding sites with the risk of SPT/recurrence in patients with early-stage head and neck cancer.

Compelling evidence has shown that miRNAs play an important role in the diagnosis, staging, progression and prognosis of a wide spectrum of tumors (11–13). These small endogenously expressed single-stranded RNA molecules (∼22 nucleotides) posttranscriptionally regulate gene expression through binding to the 3′ untranslated region (UTR) of the messenger RNAs (mRNAs) of their target genes, which leads to mRNA degradation or translation repression (14–17). It is predicted that there are >1000 human miRNAs that regulate approximately one-third of all human genes (18). Aberrant miRNAs expression and function affects a wide array of cellular pathways and leads to abnormal cellular functions (19). Thus, miRNAs are considered a group of master regulators of gene networks and play important roles in tumorigenesis.

miRNAs are mainly generated in a coordinated two-step pathway (20). First, primary miRNA (pri-miRNA) transcripts (200–300 nucleotides) are processed in the nucleus by the RNase-III enzyme RNASEN (DROSHA) and its RNA-binding partner DGCR8 (21), to form hairpin precursor pre-mRNA (22). Pre-miRNAs are then transported to the cytoplasm through the activities of RAN GTPase and Exportin 5 (XPO5) and are processed to form mature miRNAs by the endonuclease DICER1. The RNA-induced silencing complex is then formed by essential RNA processing proteins, such as EIF2C1, AGO1 and AGO2 (23). The global or specific dysfunctions of key genes in the miRNA biogenesis pathway have been associated with enhanced malignant transformation of tumors (24). In addition, single-nucleotide polymorphisms (SNPs) in miRNA biogenesis pathway genes have been found to be associated with the risks of bladder cancer, renal cell carcinoma and esophageal cancers (25–27). Furthermore, SNPs in miRNA-binding site were also shown significant association with cancer risk. A SNP in let-7-binding site in the 3′-UTR region of *KRAS* was found to significantly increase the risk of oral cancers by the overexpression of *KRAS* probably caused by weakening or abolishing the binding of let-7 (28). Overexpression of mir-24 failed to down-regulate its target gene dihydrofolate reductase (*DHFR*) gene for a SNP in its binding site localized in the 3′-UTR of *DHFR*, which resulted in resistance of methotrexate, an important drug for cancer treatment (29). Taken together, these emerging lines of evidence suggest that miRNA biogenesis proteins and miRNA-binding activity play crucial roles in cancer development and progression.

In this study, we hypothesize that genetic variations in miRNA-related pathway are associated with the risk of SPT/recurrence in patients with early-stage head and neck cancer. To test this hypothesis, we identified 235 potentially functional and haplotype tagging SNPs in miRNA biogenesis pathway genes and miRNA-binding site and evaluated their individual and joint associations with the risk of SPT/recurrence of head and neck cancer in a nested case–control study. We also conducted multiple exploratory analyses to determine the interaction effects of these variants.

Abbreviations: CI, confidence interval; HR, hazard ratio; miRNA, microRNA; mRNA, messenger RNA; MST, median survival time; SNP, single-nucleotide polymorphisms; SPT, second primary tumor; UTR, untranslated region.

Materials and methods

Study population

The subjects in this study included 150 patients with SPT/recurrence and 300 patients without SPT/recurrence derived from the Retinoid Head and Neck Second Primary Trial, which started in November 1991 and ended in June 1999. This trial recruited patients from several groups and Institutes including the Radiation Therapy Oncology Group, the University of Texas MD Anderson Cancer Center, the Clinical Community Oncology Group and the Southwest Oncology Group; the design of this trial has been reported previously (7).

These samples are matched by age (±5 years), gender and ethnicity. Detailed information on eligibility criteria and experimental design were described by Wu *et al.* (2). Demographical information was collected at registration, and blood samples were collected and kept in green-top tubes and were delivered to MD Anderson Cancer Center for cytogenetic and molecular analyses. This study was approved by all of the relevant review boards, and written informed consent was obtained from each participant.

Gene/SNP selection and genotyping

The miRNA biogenesis genes were selected through an extensive literature search. For each gene, we identified the tagging SNPs ranging from 10 kb upstream of the transcription start site to 10 kb downstream of the transcription stop site. In addition, we identified potentially functional SNPs, including non-synonymous SNPs and SNPs that may influence gene splicing or expression. These SNPs were localized in the promoter, splicing site, 5′-UTR and 3′-UTR. Tagging SNPs were selected using the LDSelect program (http://droog.gs.washington.edu/ldSelect.html), which divides all of the identified SNPs into bins based on an r^2 threshold of 0.8 and a minor allele frequency ≥ 0.01 in Caucasians. One tagging SNP was selected from each bin. Potentially, functional SNPs were selected from all two-hits in dbSNP database or HapMap validated SNPs with an Illumina designability score > 0.6 and minor allele frequency > 0.01 in Caucasians.

A custom designed panel of cancer-related genes had been built in our lab, which covered 12 main pathways and totally 998 genes. One hundred and sixty-two SNPs in 135 genes were found and predicted to be miRNA target site SNPs according to a specific database online called PolymiRTS (http://compbio.uthsc.edu/miRSNP). Genotyping was carried out using the Illumina's Infinium II iSelect platform according to the manufacturer's protocol (30).

Statistical analysis

Statistical analyses were carried out using the Stata 10.0 statistical software package (StataCorp LP, College Station, TX). Pearson's χ^2-test was used to test the differences of categorical variables such as gender and smoking status between SPT/recurrence and without SPT/recurrence groups. Student's *t*-test was used to test for differences in continuous variables. Multivariate Cox proportional hazard regression model was applied to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) on the risk of SPT/recurrence, with adjustment for age, gender, ethnicity, smoking status, tumor site, clinical stage and treatment. Three genetic models (dominant, additive and recessive) were used to evaluate the significance of SNPs. The model yielding the smallest *P* value was considered the best-fitting model. To internally validate the significant SNPs, a bootstrap resampling method was applied 100 times to samples randomly drawn from the original data set. Each time the bootstrap samples were drawn, *P* values were obtained for all three genetic models.

Table I. Association of significant miRNA SNPs and SPT/recurrence risk in patients with early-stage head and neck cancer

Gene	SNP	Position	Best fitting Model	Genotype	Event *N* (%)	No event *N* (%)	Adjusted HR (95% CI)[a]	MST (months)	*P* value	Bootstrap $P < 0.05$[b]
SMC1B	rs3747238	Binding site	Recessive	AA + AG	108 (31.12)	239 (68.88)	Reference	>93		100
				GG	39 (41.94)	54 (58.06)	1.74 (1.19–2.54)	75.8	0.004	
BCL2L2	rs1884056	Binding site	Additive	GG	59 (28.10)	151 (71.90)	Reference	>93		100
				AG	63 (36.21)	111 (63.79)	1.41 (0.98–2.03)	>93	0.066	
				AA	25 (44.64)	31 (55.36)	1.85 (1.14–3.01)	63.2	0.014	
				P for trend					0.008	
GSTM3	rs15864	Binding site	Recessive	GG + CG	127 (35.18)	234 (64.82)	Reference	>93		22
				CC	8 (20.00)	32 (80.00)	0.49 (0.24–1.00)	>93	0.049	
IL1R1	rs3917328	Binding site	Dominant	GG	131 (32.59)	271 (67.41)	Reference	>93		63
				AG + AA	16 (42.11)	22 (57.89)	1.8 (1.05–3.07)	>93	0.031	
NR1I2	rs3732360	Binding site	Additive	AA	63 (28.77)	156 (71.23)	Reference	>93		87
				AG	67 (36.61)	116 (63.39)	1.29 (0.91–1.83)	81.6	0.153	
				GG	17 (44.74)	21 (55.26)	1.96 (1.12–3.44)	>93	0.019	
				P for trend					0.018	
SSTR2	rs7210080	Binding site	Recessive	AA + AG	133 (32.20)	280 (67.80)	Reference	>93		78
				GG	14 (51.85)	13 (48.15)	1.88 (1.07–3.31)	66.9	0.028	
SUFU	rs11594179	Binding site	Dominant	GG	103 (37.45)	172 (62.55)	Reference	>93		79
				AG + AA	44 (26.67)	121 (73.33)	0.67 (0.47–0.96)	>93	0.028	
RAN	rs11061209	3′-UTR	Recessive	AA + AG	123 (32.20)	259 (67.80)	Reference	>93		77
				GG	24 (41.38)	34 (58.62)	1.6 (1.02–2.49)	81.6	0.039	
XPO5	rs2227301	3′-UTR	Recessive	GG+AG	136 (32.61)	281 (67.39)	Reference	>93		34
				AA	11 (47.83)	12 (52.17)	1.91 (1.02–3.58)	64.7	0.044	
	rs699937	Intron	Recessive	CC + CT	127 (32.23)	267 (67.77)	Reference	>93		75
				TT	20 (43.48)	26 (56.52)	1.71 (1.06–2.77)	69.7	0.028	
RNASEN	rs7735863	Intron	Dominant	AA	111 (31.09)	246 (68.91)	Reference	>93		100
				AG+GG	36 (43.37)	47 (56.63)	1.72 (1.16–2.57)	77.7	0.008	
	rs6884823	Intron	Dominant	GG	120 (31.50)	261 (68.50)	Reference	>93		99
				GA + AA	27 (45.76)	32 (54.24)	1.71 (1.11–2.65)	64.7	0.015	
	rs3792830	Intron	Dominant	TT	126 (31.90)	269 (68.10)	Reference	>93		76
				TC + CC	21 (46.67)	24 (53.33)	1.75 (1.08–2.82)	77.7	0.022	
	rs669702	3′-UTR	Dominant	GG	112 (31.55)	243 (68.45)	Reference	>93		80
				GA + AA	35 (41.18)	50 (58.82)	1.53 (1.04–2.26)	>93	0.03	
	rs639174	Intron	Recessive	CC + CT	132 (32.35)	276 (67.65)	Reference	>93		71
				TT	15 (48.39)	16 (51.61)	1.88 (1.09–3.25)	61.4	0.023	
	rs3805500	Intron	Additive	CC	49 (25.79)	141 (74.21)	Reference	>93		100
				CT	69 (37.70)	114 (62.30)	1.52 (1.05–2.20)	>93	0.029	
				TT	22 (44.00)	28 (56.00)	1.91 (1.13–3.23)	>93	0.015	
				P for trend					0.006	
	rs7719666	Intron	Additive	TT	46 (39.32)	71 (60.68)	Reference	75.8		83
				CT	73 (34.60)	138 (65.40)	0.87 (0.60–1.27)	>93	0.48	
				CC	28 (25.00)	84 (75.00)	0.59 (0.37–0.95)	>93	0.032	
				P for trend					0.034	
	rs17410035	3′-UTR	Recessive	CC + AC	141 (35.70)	254 (64.30)	Reference	>93		51
				AA	6 (13.64)	38 (86.36)	0.36 (0.16–0.83)	>93	0.016	

[a]Adjusted by age, gender, ethnicity, smoking status, clinical stage, tumor site and treatment.
[b]Internal validation of the results choosing from the best genetic model using bootstrap for 100 times.

Table II. Joint effects of unfavorable genotypes in the miRNA-related genes on the risk of SPT/recurrence

Number of unfavorable genotypes	Event, *N* (%)	No event, *N* (%)	Adjusted HR (95% CI)[a]	MST (months)	*P*-value
0–4	27 (20.9)	111 (43.0)	Reference	>93	
5–9	87 (67.4)	142 (55.0)	2.41 (1.55–3.71)	>93	8.90×10^{-5}
≥10	15 (11.6)	5 (1.9)	7.73 (3.97–15.02)	28.78	1.68×10^{-9}
P for trend					9.72×10^{-10}

[a]Adjusted by age, gender, ethnicity, smoking status, tumor site, stage and treatment.

Kaplan–Meier estimates were used to plot the event-free survival curves, and the log-rank test was used to compare the survival distributions between the genotypes.

The cumulative effects of SNPs with significant association (*P* for the best-fitting model <0.05) with the risk of SPT/recurrence were assessed by counting the number of unfavorable genotypes in each subject. Using a multivariable Cox proportional hazard regression model, HRs and 95% CIs for all groups were calculated and compared with the low-risk reference group. For the survival tree analysis, the log-rank statistics was used as node splitting criteria. Each terminal node was categorized as low-, medium- or high-risk groups based on their HR and median survival time (MST). Ten thousand bootstrap runs were conducted to construct the confidence interval for the risk estimates in the survival tree analysis. For each bootstrap run, the original dataset was replaced with new data set with the same number of observations created by random sampling. Subjects in the new dataset were classified into low-, medium- and high-risk groups according to the classification rules defined above. The confidence interval of each group was calculated from the sampling distribution of 10 000 estimated HRs. All *P* values reported in this study were two sided.

Results

Characteristics of the study population

A nested case–control study was performed including 150 patients with SPT/recurrence and 300 controls without SPT/recurrence matched by age (±5 years), gender and ethnicity. No significant differences were found between the two patient groups alcohol consumption ($P = 0.20$), radiotherapy ($P = 0.71$), surgery ($P = 0.34$) and 13-*cis*-retinoic acid treatment ($P = 0.42$). As expected, tumor site was strongly associated with SPT/recurrence: the SPT/recurrence group had fewer patients with larynx (47 versus 64%) and more patients with oral (32 versus 27%) and pharynx (21 versus 8%) than the no event group ($P = 0.00006$). SPT/recurrence was significantly associated with smoking intensity and duration. The SPT/recurrence group smoked more cigarettes yearly (55.59 ± 40.52 versus 45.31 ± 32.27 packs per year; $P = 0.007$) and for a longer duration (37.42 ± 13.63 versus 33.80 ± 4.51 years; $P = 0.02$) (2).

Risk association and survival analysis of individual SNPs

Totally, 235 SNPs were genotyped in this study (Supplementary Tables 1 and 2 are available at *Carcinogenesis* Online). Eighteen SNPs showed a significant association with SPT/recurrence after adjusting for age, gender, ethnicity, smoking status, clinic stage, tumor site and 13-*cis*-retinoic acid treatments (Table I). Among these 18 SNPs, 11 SNPs were from miRNA biogenesis pathway genes and 7 SNPs were from other genes containing miRNA-binding site. The most significant finding was rs3747238, an miRNA-binding site SNP in *SMC1B* gene. Under the recessive genetic model, compared with the wild-type containing genotypes, the variant homozygous genotype was associated with a 1.74-fold increased SPT/recurrence risk (95% CI: 1.19–2.54; $P = 0.004$). The Kaplan–Meier event-free MST was 75.8 months for the variant homozygous genotype compared with >93.0 months for the wild-type containing genotypes (log rank $P = 0.019$) (Table I, Figure 1A). For the miRNA biogenesis gene SNPs, surprisingly, eight SNPs (rs3805500, rs7735863, rs6884823, rs3792830, rs639174, rs669702, rs7719666 and rs17410035) were located in *RNASEN*. These SNPs were in weak linkage disequilibrium (data not shown). We also observed two significant SNPs in *XPO5* (rs699937 and rs2227301) and one in *RAN* (rs11061209).

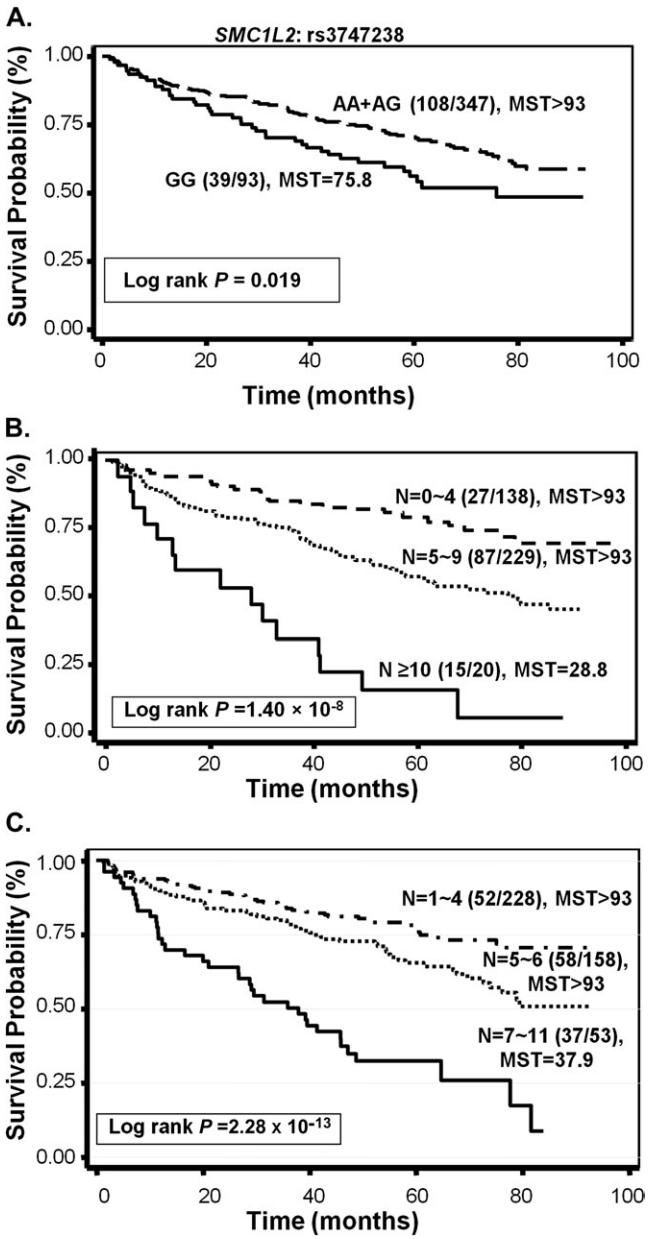

Fig. 1. (**A**) Kaplan–Meier curves of event-free survival time in early-stage head and neck cancer patients with the rs3747238 SNP. (**B**) Kaplan–Meier curve of SPT/recurrence-free survival time in early-stage head and neck cancer patient with different unfavorable genotype groups identified by cumulative effect analysis. *N*, the number of unfavorable genotypes. (**C**) Kaplan–Meier curve of SPT/recurrence-free survival time in early-stage head and neck cancer patient with different risk groups identified by survival tree analysis. The numbers in parentheses are the numbers of patients with event/total patients with the respective genotype. *N*, the number of nodes in each risk groups; MST in months.

Cumulative effects of the unfavorable genotypes

The unfavorable genotypes were then assessed for their cumulative effects by counting the number of unfavorable genotypes. We found a progressively increased risk of SPT/recurrence with an increasing number of unfavorable genotypes, and the patients could be classified into different risk groups according to their number of unfavorable genotypes. Compared with the low-risk group with 0–4 unfavorable genotypes, the HR of SPT/recurrence risk for the medium-risk group with 5–9 unfavorable genotype and the high-risk group with >10 unfavorable genotypes was 2.41 (95% CI: 1.55–3.71; $P = 8.90 \times 10^{-5}$) and 7.73 (95% CI: 3.97–15.02; $P = 1.68 \times 10^{-9}$), respectively ($P_{trend} < 0.001$) (Table II). Kaplan–Meier event-free survival curve showed that the high-risk groups had an event-free MST of only 28.78 months, much shorter than the medium- and low-risk groups (>93 months) (log rank $P = 1.40 \times 10^{-8}$ (Figure 1B).

Survival tree analysis

To explore the potential high-order gene–gene interactions between the miRNA-related gene variants, we performed a survival tree analysis using the 18 significant SNPs identified in the individual SNP analysis (Figure 2). rs17410035, a SNP in *RNASEN*, was the top splitting factor of the tree structure, and six SNPs from *RNASEN* were included in the survival tree. For each terminal node, the percentage of events, HR, *P* value and event-free MST are shown in Table III. These terminal nodes were further classified into low-risk, medium-risk and high-risk groups based on the differential risk pattern in SPT/recurrence risk (Figure 2). Using the low-risk group as reference, HR was 1.67 (95% CI: 1.13–2.45; $P = 0.0095$; 95% Bootstrap CI: 1.10–2.51) for the medium-risk group and 4.84 (95% CI: 3.11–7.51; $P = 2.45 \times 10^{-12}$; 95% Bootstrap CI: 3.04–7.37) for the high-risk group ($P_{trend} < 0.000001$) (Table IV). The event-free MST was >93 months for both low and medium groups and 37.9 months for high-risk group (log rank $P = 2.28 \times 10^{-13}$, Figure 1C).

Discussion

In this study, we reported 18 SNPs showed a significant association with SPT/recurrence risk. Seven SNPs were found in miRNA-binding site only, and the other 11 SNPs were found in the intron or 3′-UTR of miRNA biogenesis pathway genes. Surprisingly, 8 of these 18 SNPs were located in *RNASEN* (popularly known as *DROSHA*). *RNASEN* encodes a type III RNase, a main component of the microprocessor complex that functions as the catalytic subunit in the microprocessor complex and is essential to the production of pre-miRNA from primary miRNAs. DGCR8, another main partner in the microprocessor, recognizes the RNA substrate (21). Increased expression of *RNASEN*

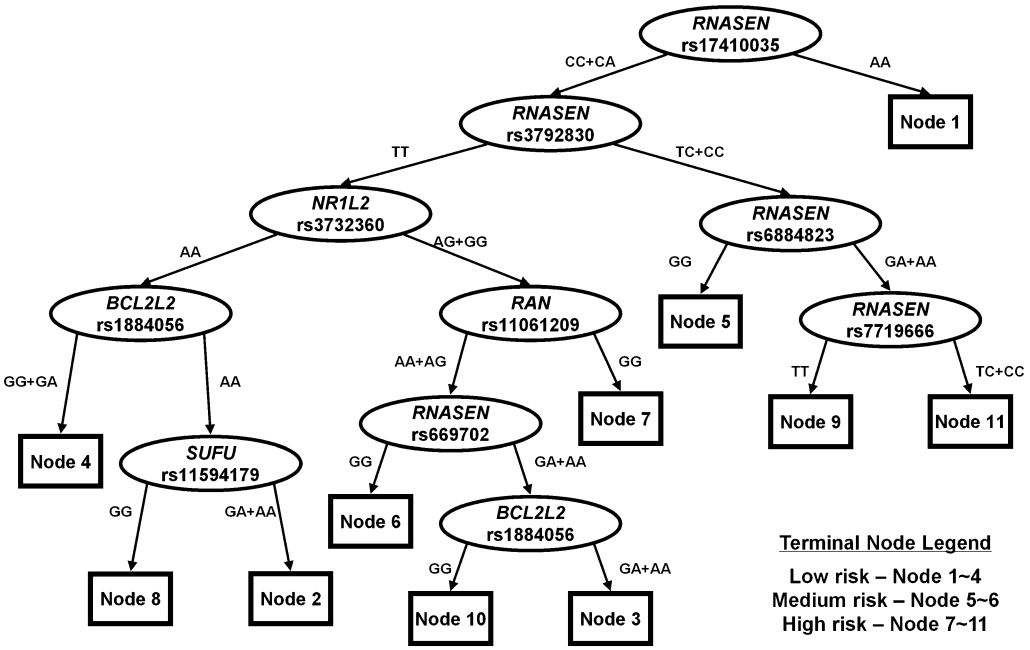

Fig. 2. Survival tree analysis using the 18 significant SNPs identified in the individual SNP analysis.

Table III. Association of SPT/recurrence risk with each terminal node identified by the survival tree analysis

Risk groups	Node	Event, *N* (%)	No event, *N* (%)	Adjusted HR (95% CI)[a]	*P* value	MST (months)
Low risk	Node 1	6 (13.64)	38 (86.36)	Reference		>93
	Node 2	3 (20.00)	12 (80.00)	1.63 (0.4–6.61)	0.491	>93
	Node 3	4 (25.00)	12 (75.00)	2.33 (0.64–8.44)	0.199	>93
	Node 4	39 (25.49)	114 (74.51)	1.87 (0.79–4.44)	0.155	>93
Medium risk	Node 5	8 (33.33)	16 (66.67)	2.96 (1.02–8.65)	0.047	>93
	Node 6	50 (37.31)	84 (62.69)	2.89 (1.22–6.84)	0.016	79.4
High risk	Node 7	6 (75.00)	2 (25.00)	6.23 (1.96–19.81)	0.0002	47.2
	Node 8	8 (66.67)	4 (33.33)	6.58 (2.19–19.81)	0.0008	39.6
	Node 9	10 (58.82)	7 (41.18)	7.18 (2.57–20.12)	0.002	37.9
	Node 10	7 (87.50)	1 (12.50)	17.2 (5.43–54.48)	1.33×10^{-6}	21.1
	Node 11	6 (75.00)	2 (25.00)	11.4 (3.54–36.85)	4.47×10^{-5}	10.1

[a]Adjusted by age, gender, ethnicity, smoking status, tumor site, stage and treatment.

Table IV. Analysis of the miRNA-related gene variants on SPT/Recurrence risk based on the results of the survival tree analysis

Unfavorable groups	Event, *N* (%)	No event, *N* (%)	Adjusted HR (95%CI)[a]	MST (months)	*P* value
Low risk	52 (22.81)	176 (77.19)	Reference	>93	
Medium risk	58 (36.71)	100 (63.29)	1.67 (1.13–2.45)	>93	0.0095
High risk	37 (69.81)	16 (30.19)	4.84 (3.11–7.51)	37.9	2.45×10^{-12}

Low-risk group: node 1–4; medium-risk group: node 5–6; high-risk group: node 7–11.
[a]Adjusted by age, gender, ethnicity, smoking status, tumor site, stage and treatment.

and *DGCR8* and other three miRNA processing genes were found to be related with deregulation expression of miRNAs in pleomorphic adenomas of the salivary gland (31). It may postulate possible effect of these significant SNPs that they may change the expression level of *RNASEN*. In addition to their direct roles in miRNA biogenesis, RNASEN and DGCR8 also regulate each other posttranscriptionally (32,33). About 80% of human miRNAs are in intronic regions, and most of these miRNAs are transcribed by their host gene promoters and require posttranscriptional splicing for maturation (34). Kataoco *et al.* (35) reported that RNASEN also played an important roles in intronic miRNA splicing. In addition, mRNA expression levels of *DICER1* and *RNASEN* have been found to be significantly associated with ovarian cancer prognosis (36). Given the critical function of *RNASEN* in miRNA biogenesis and the involvement of miRNA cancer development and progression, it is not surprising that genetic variations in *RNASEN* gene affect SPT/recurrence of head and neck cancer through regulating the function of *RNASEN* gene. The intriguing question is how these genetic variations affect *RNASEN* gene and what is the biological mechanism underlying the *RNASEN* gene variation and SPT/recurrence. The effect of *RNASEN* gene variation on cancer development and progression is probably tissue specific since *RNASEN* gene variation would affect general miRNA processing. Different cancer types have different miRNA expression signatures and the summary of functional impact of *RNASEN* gene variations would depend on the miRNA expression profile and abundance in a specific tissue. The next steps would be to evaluate the miRNA expression pattern in early-stage head and neck cancer and which miRNAs are affected by *RNASEN* gene disturbance. In this study, two of the eight SNPs (rs17410035 and rs669702) are located in the 3′-UTR region of *RNASEN* and the rest are intronic. It remains to be seen whether these SNPs affect the expression and function of *RNASEN* gene directly or they are merely tagging SNPs. Genetic fine-mapping and functional characterizations of are warranted to identify causal SNPs and their underlying molecular mechanisms.

Three additional miRNA biogenesis pathway SNPs, two in *XPO5* and the other in the 3′-UTR of *RAN*, were also significantly associated with the risk of SPT/recurrence. Consistently, genetic variants in *XPO5* and *RAN* have been found to modulate the risk of renal cell carcinoma and esophageal cancer (25,27). XPO5 is an evolutionarily conserved nuclear export factor of the importin-beta family of proteins (37). It is critical to the nuclear export of small noncoding RNAs including miRNAs (38). Knocking down *XPO5* expression led to reduced miRNA expression (39). It is possible that genetic variants in *RAN* and *XPO5* modulate miRNA transportation capacity of the cells through influencing the expression or function of their host genes. However, whether the genetic variations identified in our study have physiologic effects requires additional genetic and molecular characterizations.

In addition to SNPs from miRNA biogenesis pathway genes, there are also seven miRNA-binding site SNPs from other genes significantly associated with SPT/recurrence risk. These miRNA-targeted genes include *SMC1B*, *BCL2L2*, *GSTM3*, *IL1R1*, *NR1I2*, *SSTR2* and *SUFU*. Some of them have been reported to play important roles in carcinogenesis. The most significant SNP rs3747238 was found in *SMC1B*, which may function in chromosome structure maintenance during meiosis and mitosis (40). According to PolymiRTS, variant genotype of this polymorphism is predicted to create miRNA-binding sites for transcript targeting by miR-609 and miR-124a, which will result in lower *SMC1B* expression leading to potentially increased genome instability and greater cancer progression risk.

The *BCL2L2* gene, a member of pro-survival family, had been found to be regulated by mir-133b in lung cancer, and overexpression of mir-133b can induce apoptosis (41). Suppressor of fused homolog had been found to be a tumor suppressor in Hedgehog signaling pathway, and mutations of suppressor of fused homolog showed significant association with a childhood brain tumor (42). Genetic variations in *GSTM3* had been reported to be associated with a number of cancer types (43–46). Reporter gene assays are needed to determine whether these SNPs affect the regulation of host target genes by respective miRNAs.

We performed unfavorable genotype and survival tree analyses to elucidate the cumulative and interaction effects of these miRNA biogenesis SNPs on the risk of SPT/recurrence. We observed a trend toward an increasing SPT/recurrence risk with an increasing number of unfavorable genotypes that occurred in a dose-dependent manner. In addition, survival tree analysis indicated the presence of high-order interactions between these SNPs and identified subgroups of patients with different potentials for SPT/recurrence development. These results support the notion that the development of SPT/recurrence in curatively treated head and neck cancer patients is a polygenic process and using a pathway-based approach considering multiple factors may yield higher predictive power.

Overall, we provide the first epidemiologic evidence supporting an association between genetic variations in miRNA pathway genes and the SPT/recurrence risk for head and neck cancer patients. Nonetheless, it should be noted that although our study has one of the largest collection of head and neck cancer patients with complete SPT/recurrence follow-up data, the limited number of subjects prevented us from spliting the data into a testing and a validation set. Some of the significant SNPs are probably false positives. Future independent replication studies with larger patient populations and functional characterizations are needed to confirm these findings and explore the potential gene–gene and gene–environment interactions. Nevertheless, the presence of several significant SNPs from the same gene, the differential effect of the genotypes on event-free survival and the trend toward increased risk with increasing number of unfavorable genotypes indicate that at least some of the identified loci are true associations. These findings are consistent with the multifactorial nature of head and neck cancer and implicate the miRNA pathway in the progression of this disease, which may aid future treatment to identify high-risk populations for head and neck cancer.

Supplementary material

Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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