A Susceptibility Locus for Papillary Thyroid Carcinoma on Chromosome 8q24

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Abstract
Papillary thyroid carcinoma (PTC) displays higher heritability than most other cancers. To search for genes predisposing to PTC, we performed a genome-wide linkage analysis in a large family with PTC and melanoma. Among several peaks the highest was at 8q24, with a maximum nonparametric linkage (NPL) score of 7.03. Linkage analysis was then broadened to comprise 25 additional PTC families that produced a maximum NPL score of 3.2, P = 0.007 at the 8q24 locus. Fine mapping with microsatellite markers was compatible with linkage to the 8q24 locus in 10 of the 26 families. In the large family, a ~320 kb haplotype was shared by individuals with PTC, melanoma, or benign thyroid disease, but not by unaffected individuals. A 12 kb haplotype of 8 SNP markers within the larger haplotype was shared by 9 of the 10 families in which the 8q24 locus was compatible with linkage. The shared haplotype is located within 2 known overlapping protein-coding genes, thyroglobulin (TG) and Sre-like adaptor (SLA). Resequencing of the coding and control regions of TG and SLA did not disclose putative mutations in PTC patients. Embedded in the TG-SLA region are three likely noncoding RNA genes, one of which (AK023948) harbors the 8-SNP haplotype. Resequencing of AK023948 and one of the other RNA genes did not reveal candidate mutations. Gene expression analysis indicated that AK023948 is significantly down-regulated in most PTC tumors. The putative noncoding RNA gene AK023948 is a candidate susceptibility gene for PTC.

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Introduction
It is a relatively little discussed fact that nonmedullary thyroid carcinoma (NMTC) shows a high degree of heritability. Several large case-control studies have reported the heritability of NMTC to be one of the highest of all cancers. Papillary thyroid carcinoma (PTC) is the main form of NMTC, accounting for ~80% of all thyroid cancers. PTC is mostly sporadic; however, increasingly over the past 20 years, the occurrence of PTC running in families has been observed (1). It has been estimated that 5% to 10% of all PTC are “familial” (2, 3). To study the degree of heritability/familiality of cancers, large case-control studies have been published from Utah and Sweden (3, 4). An overall familial risk ratio (FRR) of ~2 for all cancers combined suggests a clear but modest overall contribution of predisposing genes to cancer (5). In the most common cancers, in which well-characterized subsets are inherited as regular Mendelian traits (colorectal, breast), the FRRs were mostly between 2 and 4. In contrast, the highest FRR was noted for thyroid cancer (FRR, 8.48 in Utah; 9.51 in Sweden; refs. 3, 4). Other case-control studies have measured relative risk in first-degree family members of probands with thyroid cancer specifically excluding the medullary form obtaining similar results in that PTC was among the cancers with strongest heritability (4, 6, 7). In conclusion, a major hereditary component is apparent in the predisposition to PTC.

Several linkage analyses in PTC families have been published in the past reporting at least four genomic regions (on chromosomes 1q21, 2q21, 14q31, and 19p32), which may harbor predisposing genes (8–11). However, no such predisposing genes have been identified.

In this study, we conducted linkage analyses with high density single nucleotide polymorphism (SNP) arrays in PTC families to identify candidate regions. We initially studied a large three-generation family with PTC and melanoma, then enlarged the study to comprise 26 PTC families. Evidence from linkage, haplotype sharing, and gene expression analysis implicated a putative noncoding RNA gene (AK023948 in 8q24) as a candidate gene for PTC predisposition.

Materials and Methods
The studies were approved by the Institutional Review Board at the Ohio State University, and all subjects gave written informed consent before participation.

Family samples and genomic DNA extraction. The key family in this study shown in Fig. 1 comprised individuals affected with PTC and melanoma (family #1). There were eight individuals affected with PTC; two of them had both PTC and melanoma. Among the remaining family members, two had melanoma only and two had chronic lymphocytic leukemia. An additional 10 individuals had benign thyroid disease (nodules or goiter), including one individual with goiter who also had both cutaneous and ocular melanoma, as well as breast cancer. An additional 25 families with at least 2 confirmed cases of nonmedullary thyroid cancer in close relatives were recruited. The majority (22 of 25) had 3 or more affected individuals, including a large family with 13 members affected with PTC (family #21). Family history information, pathology reports confirming the diagnosis of thyroid cancer or thyroid disease, as well as blood and tissue samples were collected from all consenting affected individuals and key unaffected individuals. The pedigrees of the 25 kindreds are provided in Supplementary Fig. S1. Genomic DNA was extracted from blood according to standard phenol-chloroform extraction procedures.

Genotyping. Genome-wide analysis of SNPs was performed by using the Affymetrix GeneChip Human Mapping 50K Array (50K_Aba_240 chip), or Affymetrix GeneChip Human Mapping 500K (Nsp 250K and Sty 250K) arrays. Sample preparation, chip hybridization, and data quality controls...
were carried out according to Affymetrix' recommendations. SNP genotype calls were made with Genechip Genotyping Analysis Software (GTYPE) 4.0 (Affymetrix) with default variables or using the BRLMM program from Affymetrix. The SNP call rate was over 92% with a P value of 0.3. The Mendelian error rate was below 0.2% and errors were removed before analysis.

**Genotyping with microsatellite markers.** Microsatellite markers were picked to span the linkage peak region on 8q24 based on the National Center for Biotechnology Information (NCBI)-uniSTS-deCODE database or markers described in the literature. The PCR primers flanking the microsatellites were obtained from the NCBI-uniSTS database or designed with the Primer3 program. Microsatellite marker designations and the PCR primer sequences are provided in Supplementary Table S1. The PCR assays were performed according to the standard PCR protocol except that one PCR primer was labeled with a fluorescent dye (HEX, FAM, or TET). Most frequently, the PCR assays were carried out using the following conditions: 2 min at 94 °C; followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C; followed by a final extension of 10 min at 72 °C. The allele analysis was performed by using ABI 3730 DNA Analyzer.

**Statistical analysis.** For genome-wide nonparametric linkage analysis, MERLIN (12) was used. Calculated allele frequencies based on genotyped individuals were used for NPL scoring. Genetic positions of NPL scores on a chromosome were indicated by using the deCODE map retrieved from Affymetrix NetAffx. The data set from family #1 was also analyzed with GENEHUNTER 2.1 (13) software with randomly selected SNPs using both nonparametric and parametric methods. Allele frequencies were calculated based on all genotyped individuals in the data set. The haplotypes were constructed by using GENEHUNTER 2.1 or MERLIN, and HaploPainter. The shared haplotype for family #1 and 9 other families was constructed based on markers in the linkage peak region. Haplotype construction and frequency estimation in healthy controls was performed with PHASE V2.1.1 program (14, 15).

**Thyroid samples and genomic DNA and RNA extraction.** Thyroid paraffin blocks were collected from 3 PTC individuals (#5, #6, #9) in family #1. Fresh-frozen thyroid samples were collected from one individual in each of 2 families (individual #1 in family #3 and individual #7 in family #8; Supplementary Table S2). Fresh snap-frozen thyroid tissue was obtained from patients with sporadic PTC undergoing surgical resection, including tumor tissue (T-PTC; n = 26) and normal thyroid tissue from the same gland (N-PTC; n = 26). Control “normal” thyroid tissue (N-Thy; n = 4) was collected from consenting individuals who had surgery because of laryngeal malignancy but no thyroid disease. Clinical data and information on the specimens will be provided upon request. These fresh frozen tissues were collected and selected for study after histologic examination. Genomic DNA was extracted by a standard phenol-chloroform procedure. Total RNA from fresh frozen tissue was extracted with TRIzol Reagent (Invitrogen). Total RNA from paraffin blocks was extracted using the RecoverAll Total Nucleic Acid Isolation kit (Ambion).

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Figure 1. Haplotypes of microsatellite markers in members of family #1. A unique haplotype (boxed) cosegregates with PTC, melanoma, and some benign thyroid diseases.

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4 http://www.ncbi.nlm.nih.gov/genome/sts/
Semiquantitative and quantitative reverse transcription-PCR. Total RNA was first treated with DNase-1 (Ambion) and then reverse transcribed to cDNA with the SuperScript First Strand Synthesis system (Invitrogen). Candidate genes and an endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were included in the same PCR reaction for semiquantitative reverse transcription-PCR (RT-PCR). All PCR assays were verified to be in the linear range by testing with different cycle numbers. Quantitative real-time PCR was performed by using an ABI PRISM 7700 DNA Sequence Detection System (Applied Biosystems) and a SYBR Green PCR kit (Applied Biosystems). The comparative threshold cycle method was used to calculate the relative gene expression. Primers for amplification of AK023948 and GAPDH are listed in Supplementary Table S1.

DNA sequencing. Genomic DNA from one PTC patient in each selected family (2 PTC patients from family #1) was used for resequencing. Deoxy-DNA sequencing of candidate genes was performed after PCR amplification of genomic DNA. The PCR primers and PCR conditions are available upon request. PCR products were directly sequenced with one of the PCR primers or primers specifically designed. Automated sequencing was performed using a PE373 DNA sequencer.

Results

Genome-wide linkage analysis in a large PTC and melanoma family. The initial genome-wide linkage analysis was conducted in a single large PTC and melanoma family (#1) using the Affymetrix 50K SNP array. Blood DNA samples from 15 individuals were genotyped, including seven affected (PTC, and/or melanoma) individuals. Separate linkage analyses were carried out using two different sets of selected SNP markers. We performed three independent parallel analyses using the following SNP sets: 1) of every 10 or 12 SNPs; 2) SNPs with the highest heterozygosity frequency; 3) a randomly selected set of SNPs. Genome-wide linkage analysis in all 26 families produced several regions positive for linkage, including the 8q24 locus. The overall linkage results in these families will be summarized and published elsewhere.

To compare the linkage results using different sets of SNP markers, we plotted NPL scores and LOD scores for chromosome 8. As can be seen in Fig. 2, the overlaid plots are similar. The combined linkage analysis in the 26 families produced a maximum NPL score of 3.2, P value of 0.007, and a maximum of LOD score of 1.3, P value of 0.02. These families could be divided into three groups: families with NPL score of ≥1 (n = 9), which are compatible with linkage to the 8q24 locus; families with NPL score of <1 (n = 10), which are likely not linked to this locus; and families with intermediate NPL scores between 0.2 and 0.7 (n = 7). One family (family #20) with an NPL score of 0.7 also produced positive evidence of linkage to the 8q24 locus by microsatellite marker genotyping (data not shown), and was included in the group of families being compatible with 8q24 linkage (n = 10), and further studied. The clinical information of these 10 families is summarized in Supplementary Table S2. Among the 10 families, 3

| Table 1. Putative susceptibility loci in a large PTC and melanoma family |
|-----------------------------|---------------------|-----------------|---------------|
| Chromosome | Max NPL* | SNP markers | Position (bp) | size (Mb) |
| 8q24 | 7.03 | SNP_A-1705523 | 133839469 | 6.3 |
| | | SNP_A-1730589 | 140993351 | |
| 12q21-24 | 6.94 | SNP_A-1696222 | 77763635 | 30.3 |
| | | SNP_A-1667888 | 108153034 | |
| 14q11 | 6.75 | SNP_A-1720255 | 19285288 | 4.2 |
| | | SNP_A-1720255 | 23471589 | |
| 6p27 | 6.5 | SNP_A-1702193 | 164689010 | 3.4 |

*Max NPL: maximum nonparametric linkage score obtained by linkage analysis with Affymetrix GeneChip Human Mapping 50K Array.
additional families (excluding family #1) had individuals with melanoma (ranging from 1-3 cases of cutaneous melanoma/family, as well as 1 case of uveal melanoma). None of these individuals had both PTC and melanoma. We noticed there was only one individual withAITD in 9 of the 10 families, including 6 cases in family #1 (Supplementary Table S2). Of the 18 individuals with AITD in these 10 families, 10 had a subsequent or concurrent diagnosis of PTC.

Haplotypes. Haplotypes for the 8q24 locus were constructed for family #1 based on markers in the central peak region. A 10-marker haplotype consisting of 6 SNPs from the 50K chip and 4 microsatellite markers was shared by 7 individuals affected with PTC and/or melanoma and 4 individuals with benign thyroid disease but not by any of the unaffected individuals who we genotyped. The haplotype was reconstructed with combined SNPs from the Affymetrix 50K chip and 250K Nsp chip and refined to a region of ~320 kb in the TG gene region (data not shown). Further haplotype sharing analysis across the 10 families showing positive linkage to 8q24 identified a smaller ~12 kb haplotype within the larger haplotype; this small haplotype was composed of 8 SNPs; the genotypes of these SNPs were obtained either from the Affymetrix 50K chip, or by direct sequencing of the region. This small haplotype was shared by at least 9 families in which the 8q24 locus was compatible with linkage (Table 2). This haplotype was seen in PTC individuals from other PTC families as well as in healthy controls. The frequency of the haplotype was 40% in our 74 Caucasian control samples.

Genes in the region. The shared haplotype is located within two known coding genes, TG and Src-like adaptor (SLA), and also covers one uncharacterized cDNA, AK023948. SLA is encoded by the antisense strand of three introns of the TG gene. The cDNA AK023948 and two other cDNAs (AK023852 and AK024366) reside in the introns of the SLA gene; These cDNAs were annotated as single-exon transcripts of 2.1 kb to 2.8 kb, which are likely noncoding RNA genes based on the very small predicted open reading frame (<100 amino acids in each), with no known paralogs or orthologs in other species. A diagram of the genomic structure of this region is shown in Fig. 3. It is noteworthy that the two cDNAs (AK023948 and AK023852) and several ESTs aligned with these cDNAs were isolated from thyroid tissue. The 8 SNP markers in the haplotype are indicated in Fig. 3.

Sequencing TG, SLA, AK023948, and AK023852. Using genomic DNA from blood we resequenced the TG, SLA, AK023948, and AK023852 genes in 7 PTC patients from 6 selected families; 1 patient from each family but 2 patients from family #1 (individuals #1 and 8 in Fig. 1). All exons and exon-intron boundary regions of the 4 genes were sequenced as well as the 2 kb promoter region of SLA isoform 1 (NM_006748) and the 2 additional exons of the 2 other SLA isoforms (NM_001045556 and NM_001045557). Numerous DNA polymorphisms were found (data not shown) but no changes suggestive of deleterious mutations were seen.

Expression analysis of genes within the locus. We have previously established gene expression profiles in PTC by using Affymetrix HG-U133 plus 2 array (17, 18). Our array data indicated that the expression level of AK023948 and AK023852 was 5 fold lower in PTC tumor versus normal thyroid tissue in approximately half of the samples tested, whereas notable differences in gene expression levels of the TG and SLA genes were not obvious between tumor and unaffected tissue (Supplementary Table S3).

To verify the array data, we performed semiquantitative RT-PCR and confirmed that AK023948 and AK023852 were significantly down-regulated in some sporadic PTC tumors as seen in samples PTC6 and PTC7 (Fig. 4A). The expression patterns of TG and AK024366 were assayed in the same sample set, with no notable alteration in tumor versus normal (data not shown; Fig. 4A). Expression of the SLA gene was very low and hard to detect in most of the samples tested but in at least 2 cases (PTC5 and PTC6) expression was slightly higher in the tumor than in unaffected tissue (Fig. 4A). Quantitative real-time RT-PCR data (Fig. 4B) showed that the expression level of AK023948 was low in 5 cases of familial PTC. There were 3 cases from the large family (kin1); samples kin1-5 and kin1-6 showed ~50% decrease (T/N ratios, 0.42 and 0.51, respectively), whereas sample kin1-9 showed a remarkable decrease (T/N ratio, 0.14). Two other familial samples (kin3-1

5 http://www.broad.mit.edu/mpg/tagger/
and kin8-2) showed significant downexpression (0.05 and 0.13, respectively). We also tested 26 cases of sporadic PTC with real-time RT-PCR (Fig. 4B). Most of the tumors (24 of 26) showed downexpression (T/N ratios from 0.02–0.8), and two samples showed overexpression (T/N ratios 1.23 and 2.36; Fig. 4B). Overall, the downexpression of the AK023948 gene was strong.

Discussion

Linkage analysis is the typical first step to localize disease genes. In PTC, at least four susceptibility loci have been proposed by others based on linkage; however, no genes have been identified. A locus on 19p32 was identified in a French family with multiple cases of an unusual form of thyroid tumors with cell oxyphilia (8). In a large Canadian family with 18 cases of multinodular goiter and 2 cases of PTC, a locus on 14q31 was proposed (9). A 1q21 locus was identified in one family with PTC and renal papillary cancer (10). Finally, a 2q21 locus was disclosed through a large study including 191 members of 80 families (11). The proportion of all families showing linkage to that locus was estimated at as high as 36%. Intriguingly, by analyzing 10 families (9 with the oxyphilic subtype), including the large French family in which the 19p32 locus was originally identified, evidence of linkage was seen not only to the 19p32 locus but to the 2q21 locus as well (19). We surmise that these previous findings suggest a high degree of genetic heterogeneity and possible multigenic inheritance in PTC.

We recruited a large three-generation family with individuals affected with PTC and melanoma and other types of cancer. Our initial genome-wide linkage analysis considering individuals with PTC and melanoma as "affected" disclosed four candidate genomic regions, on chromosomes 8q24, 6q27, 12q21-24, and 14q11. The existence of multiple linkage peaks found in one single family is hard to explain as regular genetic heterogeneity and it is possible that some of the peaks represent random noise. Epidemiologic studies have provided evidence that there is an elevated thyroid cancer risk among cutaneous melanoma survivors (20–22). PTC and melanoma may have overlapping genetic pathways in that a high prevalence of BRAF mutation has been seen in both types of tumors (23–25). It is therefore possible that undisclosed pathogenetic pathways, even susceptibility gene(s), may be shared between PTC and melanoma (25). Characteristics of familial PTC include an association with benign nodular thyroid disease. Interestingly, the linkage at the 8q24 locus in the large family was not only found in individuals affected with PTC and melanoma but also in four individuals affected with benign thyroid disease. This observation suggests that the 8q24 locus could harbor one or several susceptibility genes for PTC and other benign thyroid disease or that within this particular family, the two conditions are related.

Combined linkage analysis in 26 PTC families and additional fine mapping with microsatellite markers provided evidence compatible with linkage to the 8q24 locus in 10 PTC families, suggesting that the 8q24 locus may contribute to a sizable portion, but not the majority, of cases of familial PTC. This result again supports the concept of considerable genetic heterogeneity in PTC.

The 8q24 locus spans from 142.70 to 154.53 cM (deCODE), which overlaps with a reported locus forAITD, including Graves disease and Hashimoto's thyroiditis (HT; ref. 16). It has been previously observed that PTC is more frequent in patients with HT (26–28). There is an overlap in the morphologic features and molecular profiles between PTC and Hashimoto's thyroiditis (28). Our data imply that in some subjects, common susceptibility gene(s) residing in the 8q24 locus may act as predisposing gene(s) to both PTC and AITD.

Haplotypes constructed from markers in the 8q24 locus revealed a small 12 kb haplotype shared by at least 9 of 26 PTC families. The shared haplotype is located within two known coding genes, thyroglobulin (TG) and Src-like adaptor (SLA). Thyroglobulin is the most highly expressed protein in the thyroid gland and plays a role in cancer progression.
important roles in thyroid function. At least 35 different loss of function germline mutations in the TG gene have been identified and linked to thyroid dyshormonogenesis, specifically autosomal recessive congenital goiter and hypothyroidism (29, 30). However, it was noticed that thyroid cancer is rare in patients with TG gene mutations (31). We sequenced all of the exons and exon-intron boundary regions of the TG gene in six individuals carrying the shared haplotype and found no changes suggestive of being disease-causing. Taken together with our expression data, there is no evidence to suggest any involvement of the TG gene in PTC predisposition. Nevertheless, it is intriguing that data on genes fully contained within the TG genomic sequence suggest their involvement.

SLA is encoded by the antisense strand of introns of the TG gene, and has three isoforms. One isoform has 7 exons; the other two isoforms have 1 or 2 additional exons, respectively, functioning as 5' untranslated region. Functional studies have indicated that SLA is involved in T cell–mediated immune responses (32). We sequenced all exons and exon-intron boundaries of all isoforms and did not find deleterious mutations.

In addition to the TG and SLA genes associated with the haplotype in the 8q24 locus, there are three uncharacterized cDNAs, AK023948, AK023852, and AK024366 cDNAs (33), and numerous ESTs in this genomic region. These three cDNAs reside in introns of the SLA gene; each of them is believed to be a single exon gene ranging in size from 2.1 to 2.8 kb. Although computational analysis predicts a putative open reading frame with <100 amino acids in each, no similarities with any known proteins seem to exist. The lack of paralogs or orthologs suggests that these transcripts are unlikely to code for proteins and may be noncoding RNA genes.

We reasoned that genes predisposing to PTC should be expressed in normal thyroid tissue and their expression level might be altered (up or down) in tumor tissue. It is noteworthy that two of the genes (AK023948 and AK023852) were originally detected in normal thyroid tissue. Expression analysis revealed that AK023948 and AK023852 are indeed abundantly expressed in normal thyroid tissue and showed significant down-expression in ~50% of the PTC of tumors we tested initially. Furthermore,
real-time RT-PCR-based expression analysis in 26 sporadic and 5 familial PTC patients showed downexpression of AK023948 in 24 of 26 and 5 of 5 patients, respectively, often with dramatic reduction.

Given the likely low penetrance, high population frequency, and hypothetical gene-gene interaction model of PTC predisposition, the mechanism may be a subtle change in gene expression. However, in the absence of obvious deleterious mutations the basis of the reduced expression is unclear. Innocent-appearing DNA polymorphism(s) might be responsible for the reduced expression, and in any case, we propose that the putative noncoding RNA gene AK023948 is a candidate susceptibility gene for PTC. Taken together, we have identified a new predisposing locus in 8q24 in PTC through linkage, haplotype sharing, and gene expression analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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