รายงานวิจัยฉบับสมบูรณ์

ชุดโครงการ

ความหลากหลายทางพันธุกรรมของเอ็นซัยม์ที่เผาผลาญสารเคมีและการเปลี่ยนแปลงของยีนในเซลล์ : ความเชื่อมโยงสู่กระบวนการก่อมะเร็งและผลทางคลินิก

Inherited genetic polymorphism of xenobiotic-metabolizing enzymes and somatic gene alteration : a link to carcinogenic process and their clinical significance

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โครงการวิจัยนี้ได้รับทุนสนับสนุนจากเงินงบประมาณแผ่นดิน

มหาวิทยาลัยสงขลานครินทร์

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ส่วนที่ 2 เนื้อหา

1. ชื่อชุดโครงการ

ความหลากหลายทางพันธุกรรมของเอ็นซัยม์ที่เผาผลาญสารเคมีและการเปลี่ยนแปลงของยีนในเซลล์: ความเชื่อมโยงสู่กระบวนการก่อมะเร็งและผลทางคลินิก

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2. ชื่อโครงการย่อย

2.1 ความสัมพันธ์ของความหลากหลายของยีน ADH2, ALDH2, CYP2E1, CYP1A1, GSTM1 และ mEH กับความเสี่ยงของการเกิดมะเร็งระบบทางเดินหายใจ และทางเดินอาหารส่วนต้น

2.2 ความสัมพันธ์ของการเปลี่ยนแปลงของยีนต้านมะเร็ง p53 กับความหลากหลายของยีน ADH2, ALDH2, CYP2E1, CYP1A1, GSTM1, และ mEHX1 ในมะเร็งระบบทางเดินหายใจและทางเดินอาหารส่วนต้น

2.3 โครงการวิจัยย่อย 3: บทบาทการพยากรณ์โรคของปัจจัยควบคุมวงจรเซลล์และการตายของเซลล์ ในมะเร็งทางเดินหายใจและทางเดินอาหารส่วนต้น

3. คณะนักวิจัย และหน่วยงานต้นสังกัด

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4. บทสรุปผู้บริหาร (Executive Summary)

บทนำ

มะเร็งทางเดินหายใจและทางเดินอาหารส่วนต้น ซึ่งรวมถึงมะเร็งในช่องปากและคอหอย กล่องเสียงและหลอดอาหาร เป็นมะเร็งที่พบที่เป็นปัญหาทางสาธารณสุขสำคัญทั่วโลก เป็นมะเร็งที่พบบ่อยในสิบลำดับแรก และพบในประเทศที่กำลังพัฒนาหรือด้อยพัฒนามากกว่าประเทศที่พัฒนาแล้ว อุบัติการณ์การเกิดโรคในชายประมาณ 7.6, 3.5 และ 11.8 ต่อประชากร 100,000 คนตามลำดับ (Jemal et al, 2011) ในจังหวัดสงขลามะเร็งช่องปากและมะเร็งหลอดอาหารในชายมีอุบัติการณ์ 8.3 และ 11.5 ต่อประชากร 100,000 คน (Khuhaprema et al, 2010) ปัจจัยเสี่ยงสำคัญต่อการเกิดมะเร็งในระบบนี้ ได้แก่ การสูบบุหรี่ การดื่มแอลกอฮอล์ และเคี้ยวหมาก (Paterson et al,1996; Boonyaphiphat et al, 2002)

สารก่อมะเร็ง เมื่อเข้าสู่ร่างกายแล้วจะถูก metabolize โดยเอ็นซัยม์หลายกลุ่ม ซึ่งอาจได้ผลิตผลที่เพิ่มฤทธิ์หรือลดฤทธิ์จากสารตัวเดิม เอ็นซัยม์เหล่านี้แบ่งเป็น 2 ระยะ คือ ระยะที่ 1 ซึ่งจะเติม functional group เช่น hydroxyl group ทำให้ active intermediate metabolite และเอ็นซัยม์ระยะที่ 2 จะได้ผลิตผลที่จะกำจัดออกจากร่างกายได้ ผลิตผลที่เป็นพิษจะสามารถจับกับดีเอ็นเอ, อาร์เอ็นเอ หรือ โปรตีนในเซลล์ อันเป็นต้นเหตุทำให้เซลล์ผิดปกติไปเป็นเซลล์มะเร็ง เอ็นซัยม์ต่างๆ แต่ละคนอาจมีความสามารถในการทำงานแตกต่างกันอันเนื่องจากมีความแตกต่างบนยีน เรียกภาวะนี้ว่า ความหลากหลายของยีน (genetic polymorphism) ด้วยเหตุนี้ คนแต่ละคน จึงมีความเสี่ยงที่จะเกิดมะเร็งแตกต่างกัน ความหลากหลายของยีนที่ศึกษาในรายงานนี้ ประกอบด้วยยีนในระยะที่ 1 จำนวน 5 ชนิด คือ ยีน ADH2, ALDH2, CYP2E1, CYP1A1 และ mEH ส่วนยีนในระยะที่ 2 จำนวน 1 ชนิด คือ ยีน GSTM1

กระบวนการที่สารก่อมะเร็งทำให้เกิดมะเร็งคือการเข้าจับกับดีเอ็นเอ และทำให้เกิดการกลายพันธุ์ของยีน ซึ่งยีน p53 เป็นยีนเป้าหมายที่พบบ่อยในมะเร็งระบบทางเดินหายใจและทางเดินอาหารส่วนต้น มีรายงานว่า เนื้อเยื่อมะเร็งที่มีการกลายพันธุ์ของยีน p53 มีความสัมพันธ์กับประวัติการสูบบุรี่ของผู้ป่วย (Lazarus et al, 1996) แต่ยีนเป้าหมายที่สัมพันธ์การดื่มแอลกอฮอล์ ยังไม่มีการรายงานชัดเจน ในการศึกษานี้ จึงศึกษาความสัมพันธ์ของการกลายพันธุ์ของยีน p53 ในเนื้อเยื่อมะเร็งกับความหลากหลายทางพันธุกรรมของเอ็นซัยม์ในผู้ป่วย ผลการศึกษาจะช่วยชี้บ่งหรือเพิ่มหลักฐานความสัมพันธ์ในเชิงสาเหตุระหว่างการสัมผัสสารก่อมะเร็งในสิ่งแวดล้อมกับการเกิดมะเร็งได้

ความสามารถในการเติบโตของมะเร็ง และการตอบสนองต่อการรักษา เป็นปัจจัยชี้บ่งพยากรณ์โรคของผู้ป่วย ความสามารถดังกล่าวถูกควบคุมด้วยกระบวนการควบคุมวงจรเซลล์ และ การตายของเซลล์แบบ apoptosis โดยโมเลกุลที่มีบทบาทสำคัญ ได้แก่ p53, p16, bax, และ bcl-2 การศึกษานี้จึงศึกษาความสัมพันธ์ของการแสดงออกของโปรตีนเหล่านี้ในก้อนมะเร็งกับอัตราการรอดชีพของผู้ป่วย เพื่อหาปัจจัยชี้บ่งพยากรณ์โรค

ดังนั้นในชุดโครงการนี้ จึงเป็นการศึกษาตั้งแต่ต้นกระบวนการ จากการสัมผัสสิ่งแวดล้อม ความหลากหลายทางพันธุกรรมของเอ็นซัยม์ที่ metabolize สารเคมีนั้น การเปลี่ยนแปลงของยีนเป้าหมายในเซลล์มะเร็ง และผลลัพธ์ทางคลินิก อันจะทำให้ทราบในเชิงลึกว่าการสัมผัสสารก่อมะเร็งในสิ่งแวดล้อม ก่อให้เกิดมะเร็งได้ด้วยกลไกทางชีวโมเลกุลอย่างไร และการเปลี่ยนแปลงดังกล่าวมีผลต่อการรักษา และพยากรณ์โรคของผู้ป่วยหรือไม่อย่างไร

วัตถุประสงค์

1. ศึกษาความสัมพันธ์ของความหลากหลายทางพันธุกรรมของเอ็นซัยม์ที่ metabolize สารก่อมะเร็งในแอลกอฮอล์ ในบุหรี่ และในหมาก กับความเสี่ยงต่อการเกิดมะเร็งในระบบทางเดินหายใจและทางเดินอาหารส่วนต้น
2. ศึกษาปฏิสัมพันธ์ระหว่างยีน (gene-gene interaction) และปฏิสัมพันธ์ระหว่างยีนกับปัจจัยสัมผัสคือ การดื่มแอลกอฮอล์ การสูบบุหรี่ และการเคี้ยวหมาก (gene-environment interaction) กับการเกิดมะเร็งระบบทางเดินหายใจและทางเดินอาหารส่วนต้น
3. ศึกษาความสัมพันธ์ระหว่างความหลากหลายของยีนดังกล่าว กับการเปลี่ยนแปลงของยีน p53 ในมะเร็งของระบบทางเดินหายใจและทางเดินอาหารส่วนต้น
4. ศึกษาความสัมพันธ์ระหว่างการเปลี่ยนแปลงของยีน p53 และโปรตีนต่างๆ ที่เกี่ยวข้องกับการควบคุมวงจรเซลล์ (cell cycle) และ การตายของเซลล์ (apoptosis) ในเนื้อเยื่อมะเร็งระบบทางเดินหายใจและทางเดินอาหารส่วนต้น กับพยากรณ์ของโรค

สรุป (สรุปผลการทดลองทั้งหมดของงานวิจัยทั้งชุดโครงการ/โครงการ ทั้งตีพิมพ์แล้วและยังไม่ได้ ตีพิมพ์)

สรุปโครงการย่อย 1

การศึกษาความสัมพันธ์ของความหลากหลายของยีนกับความเสี่ยงต่อการเกิดมะเร็ง แบ่งกลุ่มผู้ป่วยเป็นสี่กลุ่มตำแหน่ง ได้แก่ มะเร็งหลอดอาหาร 377 คน มะเร็งช่องปาก 360 คน มะเร็งกล่องเสียง 229 คน และ มะเร็งแอ่งพิริฟอร์มซึ่งเป็นส่วนหนึ่งของคอหอยจำนวน 174 คน โดยมีกลุ่มควบคุมเป็นผู้ป่วยโรคอื่นๆ ที่ไม่ใช่มะเร็งซึ่งมารักษาที่โรงพยาบาลสงขลานครินทร์ในช่วงเดียวกันและมีสัดส่วนของเพศและช่วงอายุเดียวกันจำนวน 497 คน ผลการศึกษาพบว่าความหลากหลายของทุกยีนยกเว้น GSMT1 มีความสัมพันธ์กับความเสี่ยงต่อการเกิดมะเร็งในมะเร็งตำแหน่งแตกต่างกันไป โดยพบว่า ADH2*1/*2 สัมพันธ์กับความเสี่ยงที่น้อยลงต่อการเกิดมะเร็งหลอดอาหาร (OR 0.64, 95% CI 0.45-0.93); ALDH2*1/*2 สัมพันธ์กับความเสี่ยงที่ลดลงต่อการเกิดมะเร็งช่องปาก (OR 0.47, 95% CI 0.26-0.84); CYP2E1 c1/c2 สัมพันธ์กับความเสี่ยงต่อการเกิดมะเร็งแอ่งพิริฟอร์ม (OR 2.69, 95% CI 1.44-5.02) และมะเร็งกล่องเสียง (OR 2.04, 95% CI 1.29-3.20); CYP1A1 Ile/Val สัมพันธ์กับความ

เสี่ยงต่อการเกิดมะเร็งแอ่งพิริฟอร์ม (OR 0.50, 95% CI 0.27-0.93) และมะเร็งกล่องเสียง (OR 9.42 , 95% CI 4.85-18.31); EPHX1 exon 3 His/His สัมพันธ์กับความเสี่ยงต่อการเกิดมะเร็งหลอดอาหาร (OR 2.21, 95% CI 1.34-3.65) และมะเร็งช่องปาก (OR 2.37, 95% CI 1.43-3.91) และ EPHX1 exon 4 His/Arg สัมพันธ์กับความเสี่ยงต่อการเกิดมะเร็งช่องปาก (OR 0.58, 95% CI 0.38-0.88) ผลการศึกษายังพบว่า ความหลากหลายของเอ็นซัยม์และพฤติกรรมเสี่ยง (การสูบบุหรี่ และดื่มแอลกอฮอร์) มี interaction อย่างมีนัยสำคัญทางสถิติ ได้แก่ การดื่มแอลกอฮอร์กับความหลากหลายของ EPHX1 exon 3, ดื่มแอลกอฮอร์กับความหลากหลายของ mEH exon 4 และ การสูบบุหรี่กับความหลากหลาย CYP1A1 รวมทั้ง มี interaction ระหว่างยีน เช่น CYP2E1 กับ ADH2, CYP2E1 กับ GSTM1, CYP1A1 กับ EPHX1 exon4 อย่างไรก็ตาม ทิศทางความสัมพันธ์ต่อความเสี่ยงในการเกิดมะเร็งของบางยีนในการศึกษานี้ไม่สอดคล้องกับการศึกษาอื่นๆ ที่มีรายงานมาก่อน

สรุปผลโครงการ 2

ผลการศึกษา mutation ในตัวอย่างมะเร็งหลอดอาหารชนิด squamous cell carcinoma จำนวน 165 ราย พบการกลายพันธุ์ของยีน รวม 43 ตำแหน่งในตัวอย่าง 42 ราย (ร้อยละ 25. 5) ผลการวิเคราะห์พบว่าผู้ป่วยที่อายุน้อยกว่า 60 ปี มีความชุกของการกลายพันธุ์ p53 มากกว่าผู้มีอายุมากกว่า 60 ปี (ร้อยละ 38.7 เทียบกับร้อยละ 17.5, p = 0.002) ผลการวิเคราะห์ความสัมพันธ์ระหว่างการกลายพันธุ์กับความหลากหลายของยีนไม่พบความสัมพันธ์อย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตาม พบปฏิสัมพันธ์ระหว่าง การสูบบุหรี่ และ ความหลากหลายของ GSTM1 โดยพบว่า ผู้ที่สูบบุหรี่ที่มีจีโนทัยป์เป็น GSTM1 null มีความโอกาสที่จะพบ p53 mutation ในมะเร็งมากกว่าผู้สูบบุหรี่ที่มีจีโนทัยป์เป็น GSTM1 non-null (OR 2.07, 95% CI 0.87-4.89) เอ็นซัยม์ GSTM1 เป็นเอ็นซัยม์ในระยะที่ 2 เพื่อทำให้สารก่อมะเร็งลดพิษและกำจัดไปได้ ผู้ที่มีจีโนทัยป์เป็น GSTM1 non-null ไม่มีการทำงานของเอ็นซัยม์ดังกล่าว จึงทำให้มีสารก่อมะเร็งสะสมมากกว่าผู้ที่มีเอ็นซัยม์ปกติ อย่างไรก็ตาม การศึกษานี้ไม่พบปฏิสัมพันธ์ระหว่างการดื่มแอลกอฮอร์กับความหลากหลายของยีนต่อการเกิดการกลายพันธุ์ของยีน p53 ในมะเร็ง

สรุปผลโครงการ 3

ในระหว่างช่วงการศึกษา มีผู้ป่วยมะเร็งศรีษะและลำคอ 1,186 ราย แยกเป็นมะเร็งช่องปาก (oral cavity) 410 ราย มะเร็งคอหอยหลังช่องปาก (oropharynx) 357 ราย มะเร็งคอหอย (hypopharynx) 198 และมะเร็งกล่องเสียง 221 ราย ร้อยละ 66 ของผู้ป่วยอยู่ในระยะที่สามและระยะสี่ ผู้ป่วยมะเร็งกล่องเสียงมีอัตราการรอดชีพ 5 ปีสูงสุดคือร้อยละ 38 ตามด้วยมะเร็งช่องปาก ร้อยละ 25.9 มะเร็งคอหอยหลังช่องปากร้อยละ 19.2 และ มะเร็งคอหอย ร้อยละ13.4 ผลการวิเคราะห์พบว่าระยะโรคและวิธีการรักษาเป็นปัจจัยพยากรณ์โรคอย่างมีนัยสำคัญทางสิถิติ

ผลการศึกษาการแสดงออกของโปรตีนโดยวิธีอิมมูโนฮิสโตเคมีพบการแสดงออกของโปรตีน p16, p53, Bcl-2 และ Bax ร้อยละ 13.4, 44.8, 3.7, และ 65.9 ในมะเร็งช่องปาก และร้อยละ 17.9, 52.6, 21.9, 75.4 ในมะเร็งช่องปากหลังคอหอย และการแสดงออกของโปรตีน p53, Bcl-2 และ Bax ร้อยละ 58.1, 18.5 and 87.2 ในมะเร็งกล่องเสียง การแสดงออกของโปรตีน Bax ที่มาก (overexpression) สัมพันธ์กับอัตราการรอดชีพที่ไม่ดีทั้งในมะเร็งช่องปาก (Hazard ratio (HR) 1.77, 95% CI 1.04-3.01) และมะเร็งช่องปากหลังคอหอย (HR 2.21, 95% CI 1.00-4.85) ในขณะที่การแสดงออกของโปรตีน Bcl-2 ที่มากสัมพันธ์กับอัตราการรอดชีพที่ดีในมะเร็งกล่องเสียง (HR 0.23, 95% CI 0.06-0.81) ส่วนการแสดงออกของโปรตีน p16 และ p53 ไม่พบความสัมพันธ์กับการรอดชีพอย่างมีนัยสำคัญ โดยสรุป นอกจากระยะโรค และ วิธีการรักษาแล้ว การแสดงออกของโปรตีน Bax และ Bcl-2 ในก้อนมะเร็ง เป็นอีกปัจจัยที่ช่วยบ่งบอกพยากรณ์โรคในมะเร็งศีรษะและลำคออีกด้วย

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BRIEF ARTICLE

Specific intronic *p53* mutation in esophageal squamous cell carcinoma in Southern Thailand

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Abstract

AIM: To investigate *p53* mutations in esophageal cancer in a high-risk population, and correlate them with smoking, alcohol consumption and betel chewing.

METHODS: One hundred and sixty-five tumor samples of esophageal squamous cell carcinoma (ESCC) obtained from a university hospital in Songkhla province, Southern Thailand were investigated for *p53* mutations in exons 5-8, using polymerase chain reaction-single strand conformation polymorphism analysis, followed by direct sequencing. A polymerase chain reaction-restriction fragment length polymorphism (RFLP) assay was additionally used to confirm possible germline mutation in intron 6. A history of risk habits was obtained by interviews. The association between risk habits and mutation frequency was evaluated using the χ^2 test.

RESULTS: The studied specimens were from 139 male and 26 female patients with ESCC, treated at Songklanagarind Hospital. Most of the patients were smokers (86.7%) and alcohol consumers (72.73%), and 38.3% were betel chewers. Forty-three mutations of the *p53* gene were detected in 25.5% (42/165) of tumor samples. Mutations were most commonly found in exon 5 (25.6%) and exon 8 (25.6%). Mutations in the hot-spot codon 248 were found in four cases (9.3% of all mutations). G:C→C:G (30.23%), G:C→A:T (27.90%) and G:C →T:A (16.28%) were the prevalent spectra of mutations. Unexpectedly, among 10 intronic mutations, eight cases harbored a similar mutation: G→C substitution in intron 6 (nucleotide 12759, GenBank NC_000017). These were additionally confirmed by the RFLP technique. Similar mutations were also detected in their matched blood samples using RFLP and direct sequencing, which suggested germline mutations. There was no significant correlation between risk habits and *p53* mutation frequency.

CONCLUSION: A proportion of Thai ESCC patients harbored specific intronic *p53* mutations, which might be germline mutations. Further studies are needed to explore this novel finding.

Key words: Esophageal cancer; Squamous cell carcinoma; *p53* gene; Germline mutation; Mutation; Intron

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INTRODUCTION

Esophageal cancer is the eighth most common cancer worldwide, and there were 462 000 new cases in 2002[1]. It is a disease of high mortality, and ranks as the sixth most common cause of cancer death. There is a marked variation in incidence in different regions of the world; a 20-fold difference is observed between high-risk China and low-risk Western Africa. Other areas of moderately high risk are Southern and Eastern Africa, South-Central Asia, and Japan[1]. It seems that environmental carcinogens are responsible for these geographic differences and the different histological types. Tobacco and alcohol use are the main risk factors in Europe and North America[2,3], and other factors including betel chewing, hot beverages, fermented food, nutritional deficiencies or familial predisposition can be responsible for high rates in other high- or moderate-risk regions[4-7].

The incidence of esophageal cancer in Thailand is relatively low when one considers the country-wide estimates, with an age-standardized incidence rate (ASR) of 4.7 per 100 000 males in 1999[8]. However, the incidence is exceptionally high in Songkhla province in Southern Thailand, with an ASR of 8.1 per 100 000 males, which is close to worldwide incidence. In this region, oral cancer is event more prevalent, with the highest incidence (ASR 9.4 per 100 000 males) compared to other regions of the country. Most esophageal cancer cases in Thailand are squamous cell carcinomas. In our previous case-control study, alcohol consumption, cigarette smoking and betel quid chewing were found to be strong risk factors for esophageal squamous cell carcinoma (ESCC)[9].

The *p53* tumor suppressor gene is an important gene in cell cycle regulation and apoptosis. Mutations in the *p53* gene have been implicated as crucial events in the development of various cancers, including ESCC[10], and they have been identified as a vulnerable target for critical DNA damage. Analysis of *p53* mutations in various human cancers has denoted a characteristic mutational pattern that is related to specific endogenous as well as exogenous carcinogen-related agents; a finding that has given rise to the term "mutagen fingerprints" in DNA[11].

p53 mutations in ESCC from Thailand have been reported by two groups in 1997 and 2000[12,13]. However, the numbers of cases were small and the relationship between mutations and risk habits were not explicitly evaluated. Here, we analyzed the *p53* mutation profile of a larger sample set (165 cases) of ESCC using single-strand conformation polymorphism (SSCP) analysis and direct sequencing. In addition, the relationship between mutation frequency and risk habits, namely alcohol consumption, cigarette smoking and betel quid chewing, was examined.

MATERIALS AND METHODS

Patients and samples

Patients who were diagnosed with ESCC and treated at Songklanagarind Hospital during 1999-2005 were considered as candidates for the study. The study was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University, and informed consent was obtained from the patients. Data concerning detailed histories of tobacco use, alcohol consumption and betel chewing were obtained *via* face-to-face interviews using structured questionnaires. Only cases with available fresh-frozen tissue samples were included. Tissue samples were obtained from biopsy or surgical resection specimens, snapped frozen and stored at -80℃ until DNA extraction. All of the cases were primary tumors that had not been treated with radiation or chemotherapy.

Polymerase chain reaction-SSCP analysis

DNA was extracted from frozen tissues by standard methods. The tissue was digested overnight at 37℃ in lysis buffer that contained 10% SDS, 10 mmol/L Tris, pH 8.0, 10 mmol/L NaCl, 10 mmol/L EDTA and 20 μL 10 mg/mL proteinase K. DNA extraction was performed using the phenol-chloroform method and it was precipitated by 1/10 volume of 4.0 mol/L NaCl and two volumes of cold absolute ethanol.

Exons 5-8 of the *p53* gene were polymerase chain reaction (PCR)-amplified from tumor DNA, and mutations were detected by SSCP analysis. Samples that showed band shift were subjected to direct sequencing. Four sets of primer used were as follows: exon 5: TCTTCCTACAGTACTCCCCT sense, AGCTGCTCACCATCGCTATC antisense; exon 6: GATTGCTCTTAGGTCTGGCC sense, GCAAACCAGACCTCAGGCGG antisense; exon 7: TTATCTCCTAGGTTGGCTCT sense, GCTCCTGACCTGGAGTCTTC antisense; exon 8: TCCTGAGTAGTGGTAATCTA sense, GCTTGCTTACCTCGCTTAGT antisense.

PCR reactions were performed in a 50-μL volume reaction mixture that contained 0.5 μg genomic DNA, 10 pmol each primer, 100 mmol/L Tris, pH 8.3, 500 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, and 1.25 U AmpliTag Gold (Perkin-Elmer, Foster City, CA, USA). Amplification was carried out in a Perkin-Elmer 480 DNA Thermal Cycler. The PCR conditions were 95℃ for 10 min, followed by 35 cycles of 94℃ denaturation for 1 min, 58℃ annealing for 1 min, and 72℃ extension for 1 min. The final extension was conducted at 72℃ for 10 min.

For SSCP analysis, 2 μL PCR product was mixed with 5 μL 95% deionized solution that contained 0.1% bromophenol blue. The mixture was heat-denatured at 100℃ for 5 min and rapidly placed on ice. Four microliters of each denatured product of exons 5 and 8 were loaded on to the 12% polyacrylamide gel (10 cm × 8 cm × 0.75 cm) with 5.26% crosslinking (19:1 acrylamide/bisacrylamide), supplemented with 5% glycerol. For exons 6 and 7, a ratio of 49:1 acrylamide/bisacrylamide (2.04% crosslinking) was used. Electrophoresis was performed in an ice box (12℃) at 2 W and constant 10 mA for 5 h for exons 5 and 8 and 1 h for exons 6 and 7. Positive controls, which consisted of samples that had been confirmed by direct sequencing to contain the *p53* mutation, were run with each SSCP gel that was stained with silver nitrate. All positive cases were confirmed at least once by a separated PCR reaction and SSCP run.

DNA sequencing

The PCR products that showed band shift on the SSCP gel were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then directly sequenced using the Ready Reaction Dye Terminator Cycle Sequencing kit (Perkin-Elmer). The primers used in sequencing were the same as those used in the PCR. Sequencing was performed on an automated sequencer (ABI-Prism 310, Applied Biosystems, Foster City, CA, USA). All mutations were confirmed by sequencing both DNA strands.

For intron 6 mutation at nucleotide 12759, other primers, not overlapped to the mutation point, were used (forward 5'-GCCTCTGATTCCTCACTGAT-3'; reverse 5'-TAAGCAGCAGGAGAAAGCCCC-3'). This experiment was also performed on four available matched blood samples and the sequencing was performed on an automated sequencer (ABI-Prism 3130).

PCR-restriction fragment length polymorphism analysis to detect intronic G→C at nucleotide 12759

As a significant number of cases showed G→C substitution in intron 6 at the 18th base after the end of exon 6 (corresponding to nucleotide 12759 based on GenBank NC_000017), we additionally confirmed this mutation through restriction fragment length polymorphism (RFLP) analysis. This analysis was also performed on matched blood samples of these cases to investigate whether they were germline mutations.

The 181-bp PCR product was amplified using primers, forward 5'-GCCTCTGATTCCTCACTGAT-3'; and reverse 5'-TTAACCCCTCCTCCCAGAGA-3'. The PCR was performed with 100 ng genomic DNA that contained 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 2.0 mmol/L $MgCl_2$, 37.5 μmol/L each nucleotide, and 1.25 U Taq polymerase. The cycling conditions were 95℃ for 5 min, followed by 35 cycles of 95℃ denaturation for 1 min, 60℃ annealing for 1 min, and 72℃ extension for 1 min, with a final extension of 72℃ for 10 min. A 10-μL aliquot of each successful reaction was digested with 10 U *Bsa*HI restriction enzyme (New England Biolabs, Beverly, MA, USA) in 2.5 μL 10 × NEB4 buffer with 12.5 μL water at 37℃ for 2 h. The *Bsa*HI-digested fragments were separated on 10% polyacrylamide gel. A complete digestion (denoted mutation) gave 158-bp and 23-bp DNA fragments.

RESULTS

Patients and clinical data

The study included 165 tumor samples from 139 male and 26 female patients with ESCC diagnosed during 1999-2005. The mean age of patients was 63.4 years with a range of 37-91 years (Table 1). Most patients were habitual current smokers (86.7%) and drinkers (72.73%), with most of them (71.5%) reporting both habits. Habitual betel chewing was reported in 17 out of 26 females (65.4%) and in 45 out of 136 males (33.1%).

Table 1 Summary of patient characteristics

Variable	Category	No. of subjects	Frequency (%)
Sex	Male	139	84.2
	Female	26	15.8
Age (yr)	Mean, range	63.4 (37-91)	
Smoking	Never	19	11.6
	Habitual	143	86.7
	Occasional	3	1.8
Drinking	Never	33	20.0
	Habitual	120	72.7
	Occasional	12	7.3
Betel chewing	Never	78	48.1
	Habitual	62	38.3
	Occasional	22	13.6

Table 2 Location and type of mutations

Location	*n* (%)
Exon 5	11 (25.58)
Exon 6	1 (2.33)
Exon 7	9 (20.93)
Exon 8	11 (25.58)
Intron 5	1 (2.33)
Intron 6	8 (18.60)
Intron 8	1 (2.33)
Exon-intron 6	1 (2.33)
Type of mutations	
Transitions	
G:C -> A:T	8 (18.60)
G:C -> A:T at CpG	4 (9.30)
A:T -> G:C	2 (4.65)
Transversions	
G:C -> C:G	13 (30.23)
G:C -> T:A	7 (16.28)
A:T -> T:A	4 (9.30)
Tandem	
GT -> TA	1 (2.33)
Deletion	4 (9.30)

Mutation frequency and patterns

A total of 43 mutations were found in 42 tumors of the 165 samples (25.45%). The representative SSCP gels and sequencing chromatograms are shown in Figure 1.

Twenty-five mutations were missense mutations; one was nonsense, four were frameshift deletions, three were stop codons, and 10 were single base substitutions in the intron region. Mutations in coding sequences were most commonly found in exon 5 (25.58%) and exon 8 (25.58%) (Table 2). Among the 10 intronic mutations found, eight were intron 6 mutations.

Of the five major mutation hot spots of the *p53* gene (codon 175, 245, 248, 273 and 282), mutations at codon 248 were observed in four cases (accounting for 9.3% of all mutations), whereas mutations at other codons were not found.

The types of mutations are shown in Table 2. The most common type was G:C→C:G (30.23%), followed by G:C→A:T (27.90%) and G:C→T:A (16.28%). Surprisingly, eight out of 10 intronic mutations were found at the same location, that is, a G→C substitution at the

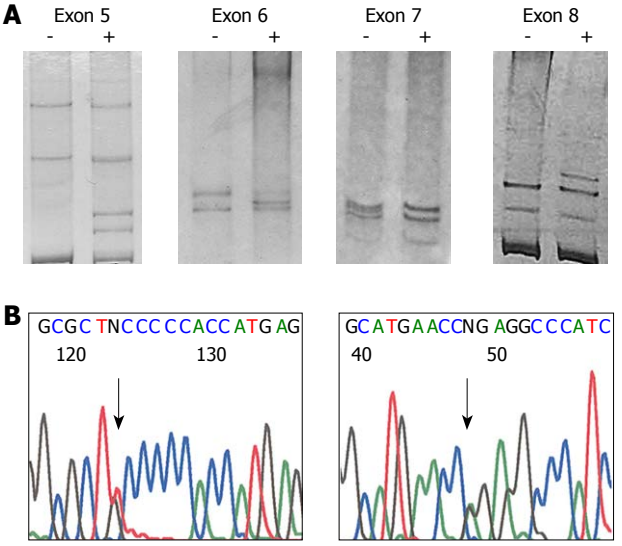

Figure 1 Single-strand conformation polymorphism analysis and direct sequencing of *p53*. A: Single-strand conformation polymorphism of *p53*, exons 5-8 in representative positive cases (+), which showed anomalous bands compared to normal bands in negative cases (-); B: Sequencing chromatograms that represent case E355 (left), which shows TGC-> TTC mutation at codon 176 (arrow) and case E106 (right), which shows CGG-> CAG at codon 248 (arrow).

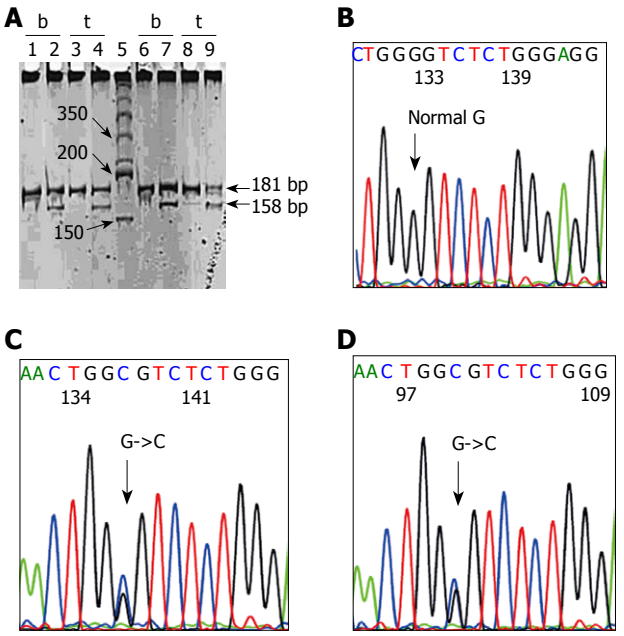

Figure 2 Restriction fragment length polymorphism analysis and direct sequencing to detect intron 6 mutation. A: Detection of the G to C substitution at base 18 after the end of exon 6 by polymerase chain reaction-restriction fragment length polymorphism using *Bsa*HI restriction enzymes. Lane 5 is the size marker. Lanes 1, 3, 6, 8 are uncut 181 bp products of blood (b) and tumor (t) samples of E189 and E199, respectively. Lanes 2, 4, 7, 9 are cut products of the corresponding samples, which show 181 bp and 158 bp fragments, which indicate the presence of a mutation. The 23-bp fragment was not detected in this gel; B-D: Sequencing chromatograms of the corresponding site. Normal sample (B); tumor (C) and blood (D) samples show G to C substitution.

18th base after the last codon of exon 6 (nucleotide 12759, GenBank NC_000017). The details of the mutations of all the cases are shown in Table 3.

Intronic G→C substitution at nucleotide 12759

As a result of the high frequency of intron 6 G→C substitution at 12759 (eight cases), we confirmed these mutations by the RFLP method and the results were similar. We further investigated whether these were germline mutations by examining their matched blood samples through RFLP and direct sequencing. Seven blood samples were available for RFLP and the results denoted a mutation in all of the cases, which suggested germline mutations (Figure 2). The DNA of only four blood samples was available for further direct sequencing and the mutations were confirmed in three out of four samples examined (Figure 2).

We also evaluated functional changes in the p53 proteins of these cases using an immunohistochemistry method (p53 antibody DO-7 clone; DakoCytomation, Carpinteria, CA, USA). Only five cases had adequate tissue for evaluation and the results showed diffuse strong expression in four cases and negative expression in one.

Mutations in relation to clinical factors and exposure

The frequency of mutations in relation to clinicopathological variables is shown in Table 4. Patients younger than 60 years had a significantly higher frequency of *p53* mutations than older patients (38.7% *vs* 17.5%, $P = 0.002$). The mutation frequency was equal in both sexes. In relation to lifestyle habits, the frequency of mutations was slightly higher in non-smokers (36.4%) than smokers (23.8%), and in non-drinkers (33.3%) than drinkers (22.5%). The mutation frequency among betel and non-betel chewers was equal (24.2% and 25.0%). However, there were no statistically significant differences in mutation frequency between exposed and non-exposed patients to all of the three habits.

DISCUSSION

The present study is the third on *p53* mutations in Thai ESCC patients. All of the samples in these three studies were from the same hospital, a university hospital in Songkhla province, Southern Thailand. These studies were conducted at different times, and the present study confirmed the findings of the previous studies and found an additional unique mutation profile.

The present study demonstrated a 25.45% (42/165) frequency of *p53* mutations. This frequency is relatively low compared to those of previous studies; however, wide variations in *p53* mutation frequencies, ranging from 17% to 80%, have been reported. These variations might be related to several factors including the sensitivity of technique used in the detection of the mutations, the length of the examined regions, and the number of cases. However, the most notable factor responsible for the frequency variation could be the difference in mutagens in different populations. A high frequency of *p53* mutation (> 50%) is usually reported in countries with a high incidence of ESCC, such as China and France[14,15], whereas lower frequency of mutation is found in low- or moderate-incidence countries[16-18]. Thailand is a moderate-risk

Table 3 ***p53*** **mutations in esophageal squamous cell carcinoma patients from Songkhla, Southern Thailand**

Case ID	Age (yr)/sex	Exon	Codon	Nucleotide change	AA change	Exposure		
						Smoke	Alcohol	Betel
E111	60/M	5	130-131	3 bp deletion	Frameshift	Yes	Yes	Yes
E303	73/M	5	134-137	10 bp deletion	Frameshift	-	-	-
E006	63/M	5	156	CGC -> CCC	Arg -> Pro	Yes	Yes	No
E271	57/M	5	158	CGC -> CTC	Arg -> Leu	Yes	Yes	No
E072	58/F	5	159	GCC -> CCC	Ala -> Pro	Yes	Yes	Yes
E200	60/F	5	161	GCC -> ACC	Ala -> Thr	Yes	Yes	Yes
E397	56/M	5	167	CAG -> CGG	Gln -> Arg	Yes	Yes	No
E379	66/M	5	168	CAC -> CTC	His -> Leu	Yes	Yes	No
E355	61/M	5	176	TGC -> TTC	Cys -> Phe	Yes	No	Yes
E014	77/M	5	176	TGC -> TAC	Cys -> Tyr	No	Yes	Yes
E022	46/M	5	184	GAT -> AAT	Asp -> Asn	Yes	Yes	Yes
E464	68/M	6	190	CCT -> CTT	Pro -> Leu	Yes	Yes	No
E259	60/M	7	228-232	21 bp deletion	Frameshift	Yes	Yes	No
E320	72/M	7	234	TAC -> TGC	Tyr -> Cys	No	Yes	Yes
E264	52/M	7	238	TGT -> TAT	Cys -> Tyr	Yes	Yes	No
E446	58/M	7	245	GGC -> CGC	Gly -> Arg	Yes	Yes	No
E106	56/M	7	248	CGG -> CAG (CpG site)	Arg -> Gln	Yes	Yes	No
E298	79/F	7	248	CGG -> TGG (CpG site)	Arg -> Trp	Yes	No	Yes
E294	52/M	7	248	CGG -> TGG (CpG site)	Arg -> Trp	Yes	Yes	No
E419	54/M	7	248	CGG -> TGG (CpG site)	Arg -> Trp	Yes	Yes	No
E022	53/M	7	249	AGG -> ATG	Arg -> Met	Yes	Yes	No
E012	74/M	8	266	GGA -> TGA	Gly -> Ter (end)	Yes	Yes	No
E231	54/M	8	272	GTG -> TAG	Val -> Ter (end)	Yes	Yes	Yes
E455	46/M	8	272	GTG -> ATG	Val -> Met	No	Yes	Yes
E449	47/M	8	278	CCT -> TCT	Pro -> Ser	No	Yes	Yes
E002	53/M	8	279	GGG -> GAG	Gly -> Glu	Yes	Yes	-
E027	54/M	8	280	AGA -> AGT	Arg -> Ser	Yes	Yes	No
E444	79/M	8	283	CGC -> CCC	Arg -> Pro	No	Yes	No
E387	74/F	8	286	GAA -> CAA	Glu -> Gln	Yes	Yes	No
E146	62/M	8	287	GAG -> TAG	Glu -> Ter (end)	Yes	Yes	No
E181	58/F	8	287	GAG -> TAG	Glu -> Ter (end)	No	No	No
E408	58/F	8	296	CAC -> CTC	His -> Leu	No	No	No
E462	74/M	Exon-intron 6		21 bp deletion	Affect splice site	Yes	Yes	No
E023	51/F	Intron 5		TGAGC -> TCTGC	-	No	No	Yes
E158	61/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Occ
E169	53/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	No
E189	60/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Yes
E199	48/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Yes
E240	61/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	-
E302	41/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Occ
E329	63/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Yes
E435	45/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	No
E409	56/M	Intron 8		ACGAG -> ACTAG	-	Yes	Yes	Yes

Occ: Occasional.

area for ESCC; therefore, the frequency can be expected to be relatively low.

Nevertheless, the two previous studies from Thailand have demonstrated higher frequency of *p53* mutations compared to the present study[12,13]. The first study by Suwiwat *et al*[12] has reported 10 mutations in eight out of 16 (50%) cases. The second study by Tanière *et al*[13] has reported 25 mutations in 23 out of 56 cases (41%). The lower frequency might represent underestimated data, whereas the higher frequency might represent overestimated data. The low frequency of mutations in the present study might have been due to various factors, among which was the fact that we used tumor samples that could have contained both tumor and non-tumor cells, in contrast to the microdissected tumor cells used in the study of Tanière *et al*[13]. With regard to the screening method used, both SSCP and denaturing gradient gel electrophoresis (Tanière study) have been reported to have comparable sensitivity[19]. However, the small number of cases examined could result in over-figured data due to sampling bias.

The present study demonstrated heterogeneous mutation types, which predominantly involved the G:C base pair. This is similar to previous Thai reports except for a relatively higher proportion of G:G to C:G transversion (30% *vs* 23%) and a lower proportion of G:C to A:T transition at CpG (9.3% *vs* 17.14%). The patterns of predominant G:A to A:T transition and G:C to T:A transversion have also been reported in high-risk areas such as China[14,20] and moderate-risk countries such as Japan and India[18,21]. This is different, however, from the high-risk area of Western Europe where a relatively

Table 4 Mutation frequency in relation to clinical factors and exposure *n* (%)

Variables	Mutant *p53*	Wild-type *p53*	*P* value
Sex			
Male	36 (25.9)	103 (74.1)	
Female	6 (23.1)	20 (76.9)	0.762
Age (yr)			
≤ 60	24 (38.7)	38 (61.3)	
> 60	18 (17.5)	85 (82.5)	0.002
Family history			
Yes	6 (37.5)	10 (62.5)	
No	33 (23.7)	106 (76.3)	0.230
Smoking			
Yes	34 (23.8)	109 (76.2)	
No/occasional	8 (36.4)	14 (63.6)	0.207
Drinking			
Yes	27 (22.5)	93 (77.5)	
No/occasional	15 (33.3)	30 (66.7)	0.155
Betel chewing			
Yes	15 (24.2)	47 (75.8)	
No/occasional	25 (25.0)	75 (75.0)	0.908

higher proportion of mutations at the A:T base pair has been reported[22].

The G:C to A:T transition accounted for 28% of all mutations in the present study. One-third of these (4/12 mutations) were G:C to A:T transition at the CpG site, and all were found at the hot spot codon 248. A G:C to A:T transition at the CpG site was thought to have resulted from spontaneous deamination of 5-methylcytosine to form thymine[23], which preferentially occurred at codons 175, 245, 248, 273 and 282 in the *p53* gene. The previous Thai studies have reported transition at the CpG site of codon 175 (one case), 273 (one case) and 248 (three cases)[12,13]. These findings suggest that codon 248 is the most common hot spot codon in Thai ESCC cases.

In reference to the G:C to A:T transition at a non-CpG site, laboratory studies have found that it is the most common mutation caused by alkylating agents, consistent with O^6-methylguanine mispairing with thymine[24]. Mutagenic alkylating N-nitrosamines in tobacco smoke might be responsible for this mutation. In China and India, dietary *N*-nitrosamines might also contribute to this mutation type[20,25]. Our previous study has demonstrated that betel chewing also is a strong risk factor for ESCC in Thailand[9]. Nitroso derivatives from areca alkaloids have been proven to be oncogenic in animal models[26]. They have been found probably to account for the predominant G:A to A:T transition in betel-chewing-related oral cancers[27]. Most of the patients in the current study had a history of drinking and smoking, as well as betel chewing, therefore, smoking and betel chewing might both contribute to the G:C to A:T transition in Thai ESCC patients. However, it is difficult to identify a specific type of mutation with a specific risk factor because the mutation patterns are considerably heterogeneous and most patients have multiple risk habits.

In the present study, we unexpectedly found a high frequency of G to C substitution at the 18th base after the end of exon 6 (nucleotide 12759, GenBank NC_000017). We additionally found that these were germline mutations because similar mutations were also found in their blood samples. We validated these results by a second method, the RFLP.

From the total of 26597 somatic mutation records in the IARC *TP53* database, version R14[28], intronic mutations have been found in 699 records, which represents 2.63% of the total mutations. G to C substitution at nucleotide 12759, similar to the present study, has been found in three cases; two were gastric lymphomas from Hong-Kong[29] and one was small-cell lung carcinoma from Russia[30]. There was a case of ESCC reported to have intron 6 G to C at nucleotide 12758. Surprisingly, this was a case from the study of Tanière *et al*[13], which was the previous study from our hospital. Looking at the details of the mutation in this published article, we found it to be GGGG→GGCG (case 9, Table II)[13], which represents a change at the 18th base after the end of exon 6 or nucleotide 12759, based on the GenBank NC_000017 reference sequence, rather than at nucleotide 12758. Surprisingly, in this case, a similar mutation was also found in the adjacent uninvolved tissue and gastric mucosa, which suggests a germline mutation. These findings suggest that intronic G to C substitution at nucleotide 12759, which might be a germline mutation, is prevalent in Thai ESCC. It should be noted that the cases included in the Tanière study would not have been included in the present study because the periods of sample collection did not overlap (1990-1998 *vs* 1999-2005).

The role of intronic base changes on the function of genes has been questioned. However, some studies have demonstrated alterations in introns or splice donors that affect the expression or function of the *p53* gene[31,32]. In particular, Lehman *et al*[32] have demonstrated functional change of the immortalized lymphoblastoid cell lines derived from familial breast cancer patients who had germline G to C substitution in intron 6 at nucleotide 13964 (or nucleotide 13274 based on the GenBank NC_000017). In addition, immunohistochemical analysis of breast tumors from these patients also has revealed high levels of mutant p53 protein, which suggests a functional mutation. Our results were consistent with this study, which confirms that cases with suspected germline G to C substitution at 12759 have a high level of p53 expression. All this evidence indicates that germline intronic G to C substitution at 12759 is prevalent and associated with inherited risk of ESCC in Songkhla, Thailand. In a recent IARC *TP53* database, version R14[28], this intronic base change has not been reported as any polymorphic sequence variation (polymorphism) or germline mutation. However, as this mutation was not investigated in healthy controls in the current study, any conclusion on the role of this mutation is still limited. Further studies to detect this mutation in healthy controls as well as in familial members of affected patients should be performed.

It is believed that *p53* mutations result from specific carcinogens[11]. In some cancers, such as those of the lung or urinary bladder, the link between risk factors, in particular smoking, and *p53* mutation frequency and/or

pattern have been consistently demonstrated[30,33]. However, such data on esophageal carcinoma are limited and inconsistent[20,34]. Consistent with some of these reports, the present study did not find any association between *p53* mutation frequency and smoking, alcohol consumption or betel chewing. Various reasons could account for the lack of association. ESCC might be associated with many risk factors. This hypothesis is supported by studies from India that have found a significant correlation of *p53* mutation frequency in ESCC with diets rich in nitrosamines[25,35]. In addition, risk of cancer development might be different among exposed individuals due to genetic polymorphism of carcinogen-metabolizing enzymes, which determine individual capacity to detoxify carcinogens. This could modify the relationship between the exposure and gene mutation. Finally, the sample size in the current study could have been too small to detect any significant association between exposure and *p53* gene mutation.

In conclusion, our results have demonstrated that the Thai population, which is in a moderate-risk area for ESCC, has *p53* mutational spectra that are likely related to specific endogenous and exogenous carcinogens. However, a statistically significant relation between the mutation frequency among exposure groups was not demonstrated. We unexpectedly found a high frequency of G to C mutation at intron 6, which might be germline mutations. Further studies are needed to explore the questions arising from the results observed.

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COMMENTS

Background

Cancer of the esophagus is prevalent in some regions of the world including Thailand. It is a dreadful disease that patients may die shortly after diagnosis. Environmental factors as well as familial predisposition have been shown to be associated with the development of this cancer, possibly *via* an alteration of the *p53* tumor-suppressor gene.

Research frontiers

Mutations in the *p53* gene have been implicated to be critical events in the development of various cancers. Significant association between specific exposures and the *p53* mutations has been evident in some cancers, but the data in esophageal squamous cell carcinoma are limited.

Innovations and breakthroughs

The mutation profiles identified are consistent with most previous reports. The mutation types, G:C to C:G (30.2%), G:C to A:T (27.9%) and G:C to T:A (16.3%) were prevalent and likely to be associated with combination of exposures. Exceptionally, a unusually high frequency (8 from 42 cases) of intron 6 mutation (G to C substitution) at nucleotide 12759 was found and they were proofed to be germline mutations.

Applications

The results indicated that a proportion of esophageal cancer in this region is heritable. Further study is to be conducted to identify this specific germline mutation in healthy population and in familial members of the patients. The information would be valuable for designing diagnosis and preventive intervention in high-risk population.

Terminology

An intron is a region within a gene that is not translated into protein. It is transcribed to pre-mRNA and subsequently removed by a process called splicing. A germline mutation is a heritable variation in the lineage of germ cells. Mutations in these cells are transmitted to offspring while those in somatic cells are not. Germline mutations play a key role in genetic diseases and also in certain types of cancer.

Peer review

The authors found intronic *p53* mutation in esophageal squamous cell carcinoma in Southern Thailand, which was considered as a germline mutation. This is a novel finding and interesting.

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The Prognostic Value of p53, Bcl-2 and Bax Expression in Laryngeal Cancer

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Objective: *Determine the prognostic value of p53, Bcl-2 and Bax expression in cancer of the larynx.*
Material and Method: *Ninety-four patients diagnosed with laryngeal squamous cell carcinoma were analyzed for 5-year overall survival in relation to immunohistochemical expression of p53, Bcl-2, and Bax proteins.*
Results: *The present study included 86 males and eight females with a mean age of 65.1 years. Half of the patients (51%) were in stages III and IV. Radiation (44.7%) and radiation plus surgery (40.4%) were the main treatments. The frequency of p53, Bcl-2, and Bax expression was 58.1%, 18.5%, and 87.2%, respectively. The 5-year overall survival rate was 49.7%. Univariate analysis revealed that T-stage, N-stage and treatment were significantly associated with 5-year overall survival. In the multivariate Cox regression, T-stage, treatment, and Bcl-2 expression were significantly associated with survival. Positive Bcl-2 expression was associated with better survival (Hazard ratio 0.23, 95% CI 0.06-0.81).*
Conclusion: *The positive Bcl-2 expression is an independent prognostic marker in laryngeal squamous cell carcinoma.*

Keywords: *Larynx cancer, p53, Bcl-2, Bax, Prognostic marker*

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Head and neck cancers are the sixth most common cancers in the world with a global incidence of 700,000 cases per year[1]. Squamous cell carcinoma constitutes the majority of tumor types of head and neck squamous cell carcinoma (HNSCC), including oral cavity, larynx, and pharynx respectively.

In most reports, more than half to two-thirds of the HNSCC patients are at advanced stages of disease at presentation, and this contributes to poor survival of the patients. Even with the advances in medical technologies, the survival outcomes of HNSCC have only subtly increased during the past two decades[2]. Therefore, identification of biological markers to predict a patient's clinical outcome is crucial for effective treatment planning.

Apoptosis and cell cycle control are the two intimately linked molecular pathways involved in carcinogenesis and progression of cancer cells. p53 protein, a product of TP53 tumor suppressor gene, plays a role both in cell cycle control and apoptosis by inducing growth arrest and initiating apoptosis after exposure to DNA damage[3]. Mutation of the TP53 gene results in an abnormal protein that can be detected by a routine immunohistochemical technique. The p53 protein regulates apoptosis via transcriptional activation of Bax and suppression of Bcl-2[4]. Bax and Bcl-2 are important members of the Bcl-2 family proteins, which play roles in the regulation of apoptosis[5]. Apoptotic cell death is an important mechanism for radiation response. Therefore, the ability of tumor cells to confer apoptosis is thought to relate to treatment success or failure.

Many studies have evaluated the prognostic value of p53, Bcl-2, and Bax or other apoptotic proteins in HNSCC, with the largely conflicting results. Therefore, the authors simultaneously evaluated the expression of p53, Bcl-2, Bax in a large series of LSCC.

Material and Method

The present study was reviewed and approved by the institutional ethics committee. The studied subjects included 94 patients who sought treatment at Songklanagarind Hospital, Department of Otolaryngology Head and Neck Surgery, Faculty of Medicine, Prince of Songkla University, with primary

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laryngeal squamous cell carcinoma (LSCC), from January 2002 to December 2004. The demographic and clinical data were reviewed, as well as the extent of tumor, lymph node involvement, and stage determination, classified according to the International Union against Cancer (UICC) classification, fifth edition, 1997.

Surgical resection or radiotherapy was given for patients in stage I and early stage II cancer, combined surgery and radiotherapy was for late stage II to stage IV disease, and radiochemotherapy for advanced cancer with an unacceptable outcome of surgical morbidity. Radiotherapy alone was considered for palliative treatment in patients with advanced disease who were not physically fit for combined therapy.

Death information was obtained from the census registration data of the Department of Provincial Administration, Ministry of Interior, where the data is linked nationwide. Patients not found dead in this database up to December 2009 were designated as alive in this present study cohort.

Immunohistochemistry and evaluation

Immunohistochemistry was performed on paraffin sections. Antigen retrieval was accomplished by immersing slides in Tris EDTA buffer pH 9 in a pressure cooker at 95°C for 4 minutes. Endogenous peroxidase was blocked by 3% hydrogen peroxide. The slides were incubated with primary antibodies against p53 (clone DO7, DakoCytomation; dilution 1:100), Bcl-2 (clone bcl-2/100/D5, Novocastra; dilution 1:80) and Bax (polyclonal, DakoCytomation; dilution 1:150). The slides were then incubated with EnVision for 30 minutes followed by color development using diaminobenzidine and counterstained with hematoxylin. The sections of esophageal squamous cell carcinoma that were known to be strongly positive for p53 expression were used as positive controls. Sections of endometrial hyperplasia were used as positive controls for Bcl-2 and Bax. Infiltrating lymphocytes were also used as internal positive controls for Bcl-2 and Bax expression.

The percentage of positive stained tumor cells was estimated overall by assessing the whole slide. The presence of more than 5% of Bax and Bcl-2 and 10% of p53 was considered positive expression. Intensity of staining was recorded as weak, moderate and intense.

Statistical analysis

The correlation between clinicopathological variables and protein expression was assessed by Chi-squared test. Five-year overall survival (OS) was obtained by the Kaplan-Meier method. The log-rank test was used to compare differences in survival among subgroups of each variable. Cox proportional hazards regression was performed to obtain independent prognostic factors for survival. The 5% level of significance was considered statistically significant. As no significant difference between using percentage and intensity of protein expression was found, the percentage of expression was used in all analyses. All analyses were carried out using statistical package STATA version 6.0.

Results

The present study included 86 males (91.5%) and eight females (8.5%). The mean age was 65.1 years, with range of 35 to 87. About half of the patients (54.2%) were in advanced stages (stage III and IV) at presentation; among the distribution of stage I/II/III/IV/unknown were 25.5/12.8/23.4/30.8/7.5 respectively. Most of them received radiation (44.7%) or radiation plus surgery (40.4%). However, a number of 10 patients (11%) were lost to follow-up before any treatment was given.

The frequency of p53 expression was 58.1% and most showed strong nuclear staining, and the frequencies of Bcl-2 and Bax expression were 18.5% and 87.2%, respectively with varied intensities from weak to intense.

Median survival time of the patients was 42.7 months. The 5-year overall survival (OS) rate was 49.7% (95% CI 38.8-59.6). Univariate analysis by Kaplan-Meier method and log-rank test revealed that T-stage, N-stage and treatment were significantly associated with 5-year OS. Bcl-2 expression is associated with a higher 5-year OS (72%) compared to a negative Bcl-2 expression (44.4%) with marginal statistical significance ($p = 0.06$), as in Table 1, whereas p53 and Bax expression were not associated with survival.

In the multivariate Cox regression, T-stage, treatment and Bcl-2 remained significant. Bcl-2 expression was independently associated with better survival (Hazard Ratio 0.23, 95% CI 0.06-0.81).

Discussion

The current study showed that Bcl-2 expression was significantly associated with survival, while p53 and Bax expression were not. The several studies showed that p53 expression was not related with the clinical outcomes[6,7], and inconclusive by a meta-analysis[8]. The present study did not show any

Table 1. Univariate and multivariate analyses for clinical parameters and protein expression in relation to overall survival (n = 94)

Variables	5-yr overall survival (%)	p-value[a]	Hazard ratio	95% CI	p-value
T stage					
T1	74.7	0.01	1		
T2	48.0		4.67	1.49-14.59	0.01
T3-4	36.9		9.14	3.06-27.35	0.00
N stage					
N0	58.2	0.03	1		
N1	37.5		0.87	0.27-2.77	0.83
N2-3	33.3		0.91	0.40-2.06	0.82
Treatment					
Untreated	0	0.00	1		
Surgery	66.7		0.14	0.01-1.27	0.08
Radiation	47.92		0.25	0.09-0.73	0.01
Surgery + radiation	61.8		0.08	0.02-0.27	0.00
P53 expression					
Negative	44.2	0.59			
Positive	54.6		0.65	0.32-1.32	0.24
Bcl-2 expression					
Negative	44.4	0.06			
Positive	72.1		0.23	0.06-0.81	0.02
Bax expression					
Negative	58.3	0.52	1		
Positive	48.0		1.07	0.33-3.54	0.91

[a] log-rank test

statistical significance of Bax expression, consistent with other studies[9,10].

Bcl-2 contributes to neoplastic cell expansion by preventing cell turnover caused by the physiological cell death mechanism. Over-expression of the Bcl-2 also prevents cell death induced by nearly all cytotoxic anticancer drugs and radiation[11]. Most of these studies reported a lack of association of Bcl-2 expression with clinical outcomes; however, a few studies demonstrated a positive association[12,13] or even a reverse association (Bcl-2 expression associated with better prognosis)[14,15], as the present study. Some authors have demonstrated lower Ki-67 labeling index and/or apoptotic labeling index in Bcl-2+ foci/tumor and these tumors are associated with a better prognosis[16,17].

In conclusion, the positive Bcl-2 expression is an independent prognostic marker in laryngeal squamous cell carcinoma.

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Potential conflicts of interest

None.

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คุณค่าการพยากรณ์ของการแสดงออกโปรตีน p53, Bcl-2 และ Bax ในมะเร็งกล่องเสียง

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วัตถุประสงค์: *เพื่อระบุหาคุณค่าการพยากรณ์ของการแสดงออกโปรตีน p53, Bcl-2 และ Bax ในมะเร็งกล่องเสียง*
วัสดุและวิธีการ: *ผู้ป่วยจำนวน 94 ราย ผู้เป็นมะเร็งกล่องเสียงชนิดเซลล์แบน ได้วิเคราะห์การอยู่รอดภาพรวม 5 ปี มีความสัมพันธ์กับการแสดงออกของโปรตีน p53, Bcl-2 และ Bax ด้วยวิธี immunohistochemistry*
ผลการศึกษา: *การศึกษานี้มีผู้ป่วยเป็นชาย 86 ราย และหญิง 8 ราย อายุเฉลี่ย 65.1 ปี ผู้ป่วยครึ่งหนึ่ง (ร้อยละ 51) เป็นระยะที่สามและสี่ การรักษาโดยวิธีรังสีรักษา (ร้อยละ 44.7) และรังสีรักษากับการผ่าตัด (ร้อยละ 40.4) เป็นการรักษาหลัก อัตราการรอดชีวิตภาพรวม 5 ปี เท่ากับร้อยละ 49.7 ความชุกของการแสดงออกของโปรตีน p53, Bcl-2 และ Bax เท่ากับร้อยละ 58.1, 18.5 และ 87.2 ตามลำดับ พบว่าการรอดชีวิตภาพรวม 5 ปี มีความสัมพันธ์อย่างมีนัยสำคัญกับระยะขนาดก้อนมะเร็ง ระยะต่อมน้ำเหลือง และการรักษา ด้วยการวิเคราะห์ตัวแปรเดี่ยว เช่นเดียวกับ ระยะขนาดก้อนมะเร็ง การรักษา และการแสดงออก Bcl-2 ด้วยการวิเคราะห์พหุตัวแปรแบบ Cox การแสดงออก Bcl-2 ได้ผลบวกสัมพันธ์กับพยากรณ์โรคที่ดี (Hazard ratio 0.23, 95% CI 0.06-0.81)*
สรุป: *การแสดงออกของโปรตีน Bcl-2 ได้ผลบวกเป็นปัจจัยพยากรณ์ของมะเร็งกล่องเสียง*

RESEARCH COMMUNICATION

Survival and Prognostic Factors of Different Sites of Head and Neck Cancer: An Analysis from Thailand

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Abstract

Background: Head and neck cancers are prevalent in Thailand, in particular in the southern region of the country. However, survival with a large data set has not been reported. The purpose of the present study was to evaluate the survival figures and the prognostic factors in a cohort of patients treated in a university hospital located in the south of Thailand. Patients and Methods: Consecutive new cases of primary carcinoma of the oral cavity, oropharyx, hypopharynx and larynx, treated at Songklanagarind Hospital during 2002 to 2004, were analyzed. The 5-year overall survival rates were obtained by the Kaplan-Meier method. Prognostic factors were identified through multivariate Cox regression analysis. Results: A total 1,186 cases were analyzed. Two-thirds (66.6%) of the cases were at advanced stage (stage III & IV) at presentation. The five-year overall survivals for the whole cohort, oral cavity, oropharynx, hypopharynx and larynx were 24.1%, 25.91%, 19.2%, 13.4%, 38.0% respectively. Stage and treatment type were strong prognostic factors for all sites. An age ≥ 80 years was associated with poor survival in oral cavity and larynx cancer. Conclusions: The results revealed remarkably poor outcomes of the patients in the series, indicating a strong need to increase the proportion of early stage presentations and maximize the treatment efficacy to improving outcomes. Very old patients are of particular concern for treatment care of oral cavity and larynx cancer.

Keywords: Head and neck cancer - upper aerodigestive tract - cancer - survival - prognosis - Thailand

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Introduction

Head and neck cancer (HNCA) is among the major public health problem worldwide, especially in developing countries (Jemal et al., 2011). Oral cavity cancer is the most common among the various anatomical subsites. In Thailand, HNCA is common in the southern region et al., 2010). The age-standardized incidence rate (ASR) of oral cavity cancer in males in Songkhla province, southern Thailand, is among the highest incidences (8.3 per 100,000), slightly lower than the eastern region of the country but it is considerably higher than the average global incidence in both developed (6.9 per 100,000) and less developed areas (4.6 per 100,000) (Jemal et al., 2011). Head and neck cancer is known to be associated with high morbidity and mortality. Mortality from oral cancer averages less than half the incidence (Jemal et al., 2011). The 5-year survival rate of HNCA has subtly increased during the past two decades, in contrast with the advances in treatment modality (Carvalho et al., 2005). This figure is largely a result of the advanced stage of the disease at diagnosis which, in turn, limits or causes suffering from treatment. In addition, the survival and prognostic factors of different anatomical sites are reported to differ. The 5-year survival rates fall between 40 to 60%, based on the site (Woolgar et al., 1999; Pericot et al., 2000). These rates are likely the result of multiple factors, including the stage of disease at the time of diagnosis, treatment modalities, and the site-specific morbidity associated with each treatment.

Although the survival rate of HNCA has been frequently cited as subtly changing during the past years, an analysis of survival based on the Surveillance, Epidemiology and End Results (SEER) database in the United States form 1973 to1997 revealed a significant improvement of the 5-year survival rates of some specific sites, including the nasopharynx, oropharynx and hypopharynx (Carvalho et al., 2005). Even though HNCA is among the five leading cancers in Thailand, the survival figure of the disease has been rarely reported in the literature. Therefore, we have analyzed the overall survival rates and clinicopathological prognostic factors of a cohort of HNCA patients treated at a university hospital located in the south of Thailand. A special focus of this study is a site-specific analysis, including the oral cavity, oropharyx, hypopharynx and larynx. Our study has provided the current situation for

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survival figures and treatment results of HNCA in our institution, which approximately represents the survival figure in the population.

Materials and Methods

Patients and clinical information

The study included all new patients with primary carcinoma of the four anatomical sites in the head and neck region, including the oral cavity (ICD10, C00-C06), oropharyx (C09-C10), hypopharynx (C12-C13) and larynx (C32), who sought treatment at Songklanagarind Hospital from January 2002 to December 2004.

Case finding and clinicopathological data as well as follow-up information were prospectively collected from hospital and pathological records by a trained nurse of the Department of Otorhinolaryngology, Faculty of Medicine, Prince of Songkla University. For patients who were treated by the Department of Surgery, the data was retrieved from the Cancer Registry Unit of Songklanagarind Hospital which is responsible for registering all cancer cases in the hospital. Patients diagnosed in either our hospital or at other hospitals and referred for treatment were included. Data on stages was missing in patients who did not come for further investigation or treatment after diagnosis.

Primary tumors, lymph node involvement and stage determination were classified according to the International Union Against Cancer (UICC) classification, Fifth Edition, 1997. Pretreatment staging and evaluation included complete history taking, physical examinations and investigations. Physical examinations included a complete otolaryngologic endoscopic examination under local or general anesthesia. Plain film of the chest and a CT scan of the head and neck were done in most cases for primary, nodal and distant metastasis evaluation. Complete blood count, blood urea nitrogen, serum creatinine and liver function tests were basic laboratory workups.

Treatment protocol

All new cancer patients were subject to treatment planning based on a multidisciplinary tumor conference. Performance status based on the Eastern Cooperative Oncology Group (ECOG) (Oken et al., 1982) was assessed for a treatment decision. Either surgery or radiotherapy was the only modalities in stage I and early stage II cancer. Combined surgery and radiotherapy was chosen for patients with late stage II, stage III and stage IV cancer. Radiochemotherapy was the treatment of choice for advanced stage cancer with an unacceptable outcome of surgical morbidity and for patients who had an ECOG scale of 0-2. Radiotherapy alone was considered for palliative treatment in patients with advanced stage disease and who were not physically fit for combined therapy.

Radiation protocol

Patients were treated with a 6 MV linear accelerator or Cobalt-60 machine. The position and treatment fields were determined by conventional simulation. The daily conventional fractionation of 2 Gy per fraction was used to deliver a radiation dosage of 66-70 Gy in 33-35 fractions over 45-47 days for the primary tumors and macroscopic lymph node. The adjacent non-tumor area or the negative surgical margin was treated with 50-54 Gy in 25-27 fractions. The spinal cord was shielded after 40-44 Gy, then the electron beams were used for the remaining optimal radiation dosage.

Death information

Death information was retrieved from the Department of Provincial Administration, Ministry of Interior. In Thailand, death has to be reported to the local registration office within 24 hours. Census registration data is linked nationwide and can be assessed with authorized permission. The Cancer Registry Unit of the hospital updates the death information from the census registration data twice yearly. Patients not found dead in this database up to December 2008 were designated as alive in this study cohort. The cause of death was classified as related or unrelated to cancer.

Statistical analysis

Statistical analysis was carried out using the statistical package STATA version 6.0. Two-year and 5-year overall survival of the whole cohort and of each anatomical site were obtained by the Kaplan-Meier method and the significance of differences between curves as classified by variable category was evaluated by the log-rank test as univariate analysis. The starting date of the analysis was set at the date of definite clinical diagnosis usually confirmed by pathological reports. The endpoint was the date of death updated most recently, during October to December 2008. Patients who were still alive at this time were considered as censored cases. Multivariate Cox proportional hazards regression was performed to investigate the relationship between clinicopathological characteristics and survival. A p value less than or equal to 0.05 was considered statistically significant.

Results

During 2002 to 2004, there was a total of 1,186 cases of HNCA, including 410 oral cavity cases (34.6%), 357 oropharynx cases (30.1%), 198 hypopharynx cases (16.7%) and 221 larynx cases (18.6%). The histological type of the tumors was mostly squamous cell carcinoma (94.8%).

Patient characteristics for all cases and each site are shown in Table 1. The mean age of the patients was 65.43 years and equal for all sites. Approximately 90% of patients were males, except in oral cavity where males constituted 58% of the cases. Two-third (66.61%) of the cases presented with advanced stage (stages III & IV) cancer. Hypopharynx cancer had the highest proportion of patients with advanced stage (84.85 %), while larynx cancer had the smallest proportion (58.37%). Radiation alone was the most common treatment for all sites (32.7-51.8%) while a minority of patients receiving surgery alone (1.4-8.78%). Nearly one-third of the patients (337 cases, 28.41%) received no treatment. These untreated patients were slightly older than the treated group (68.19 versus 64.33 years) and the stages of disease at diagnosis were stage I-II, 20.18%; stage III-IV, 64.99% and

unknown stage, 14.84% compared to 30.86%, 67.26% and 1.88%, respectively, in the treated patients (data not shown).

For the whole series, 889 patients (74.96%) were dead at the end of 2008. The overall median survival time was 24.08 months with 2-year and 5-year overall survival (OS) rates of 37.76% and 24.08% respectively. The 5-year OS among the four sites was significantly different (p value of log-rank test 0.000). Larynx cancer had the highest 2-year and 5-year OS (57.36% and 38.00%), followed by oral cancer (36.36% and 25.91%), oropharynx (32.96% and 19.24%) and hypopharynx (27.41% and 13.43%) (Figure 1). The survival curves orderly declined from higher to lower stages. The survival curve of each stage is clearly separated in oral cavity cancer with some overlapping in other cancer sites. Five-year OS according to clinicopathological variables are present in Table 2. Univariate analysis using log-rank test revealed that stage and treatment were consistently significantly

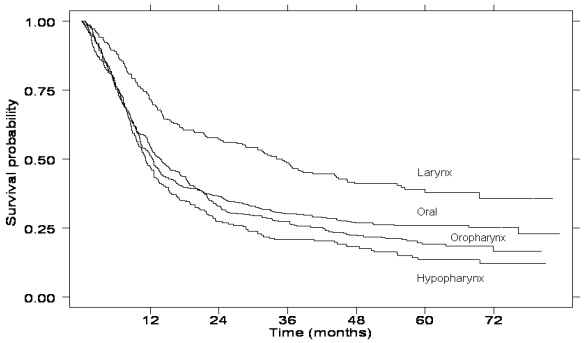

Figure 1. The Overall Survival According to the Four Anatomical Sites

Table 1. Patient Characteristics of All Cases and by the Four Anatomical Sites

Variables	Number of cases (%)				
	All cases	Oral cavity	Oropharynx	Hypo-pharynx	Larynx
Age (mean, SD)	65 (12)	65 (13)	65 (11)	66 (11)	66 (11)
Gender:					
Male	55 (81)	238 (58)	328 (92)	184 (92)	205 (93)
Female	31 (20)	172 (42)	29 (8)	4 (7)	6 (7)
Stage:					
I	3 (13)	61 (15)	33 (9)	(4)	1 (23)
II	7 (15)	75 (18)	63 (18)	9 (5)	0 (14)
III	6 (22)	82 (20)	89 (25)	46 (23)	49 (22)
IV	4 (44)	163 (40)	159 (45)	122 (62)	80 (36)
Unknown	6 (6)	9 (7)	3 (4)	3 (7)	1 (5)
Treatment:					
Surgery	9 (4)	36 (9)	5 (1)	2 (1)	6 (3)
RT a	14 (43)	134 (33)	185 (52)	97 (49)	98 (44)
Surgery	286 (24)	114 (28)	65 (18)	48 (24)	59 (27)
No	37 (28)	126 (31)	102 (29)	51 (26)	58 (26)
Differentiation:					
Well	416 (35)	214 (52)	94 (26)	39 (20)	69 (31)
Moderate	303 (26)	77 (19)	120 (34)	59 (30)	47 (21)
Poor	136 (12)	19 (5)	60 (17)	34 (17)	23 (10)
Unknown	331 (28)	100 (24)	83 (23)	66 (33)	81 (37)

* 'Abbreviations: RT, radiotherapy

Table 2. Five-Year Overall Survival Rates, According to Clinicopathological Variables

Variables	All cases	Oral cavity	Oro-pharynx	Hypo-pharynx	Larynx
Age:					
< 60	26.8	29.6	20.3	11.1	45.6
60-69	29.3	29.3	25.3	20.9	42.7
70-80	20.4	24.2	13.7	11.3	33.3
> 80	12.0	14.9	8.1	5.9	10.5
p	0.000[a]	0.134	0.056	0.383	0.000
Gender					
Male	23.1	24.2	18.9	13.4	49.1
Female	28.2	28.3	22.6	14.3	37.4
p	0.437	0.635	0.811	0.542	0.133
Stage					
I	53.7	45.7	47.7	0	69.8
II	34.5	37.5	26.2	44.4	38.7
III	24.1	20.1	19.7	23.8	37.9
IV	12.8	14.8	10.5	7.2	21.5
Unknown	17.5	32.2	15.4	0	0
p	0.000	0.000	0.000	0.000	0.000
Treatment					
Surgery	59.8	61.7	80.0	0	50.0
RT	20.8	18.6	17.8	11.8	38.6
Surgery	38.5	32.7	38.7	29.7	56.4
Untreated	11.4	17.1	6.0	0	16.8
p	0.000	0.000	0.000	0.000	0.000
Differentiation					
Well	27.8	27.3	21.2	12.8	46.2
Moderate	20.3	25.1	16.8	14.5	27.2
Poor	22.9	0	20.4	29.8	33.5
Unknown	23.2	27.2	19.0	5.1	37.7
p	0.635	0.055	0.751	0.024	0.242

* '[a]p value, log-rank test

Table 3. Multivariate Cox Regression Analysis of Oral Cavity and Oropharynx Cancer

Variables	Oral cavity		Oropharynx	
	HR (95% CI)	p	HR (95% CI)	p
Age:				
<60	1		1	
60-69	1.01 (0.73-1.39)	0.94	1.00 (0.73-1.38)	0.96
70-79	1.06 (0.78-1.44)	0.70	1.41 (1.04-1.93)	0.03
> 80	1.57 (1.09- 2.27)	0.02	1.04 (0.67-1.64)	0.84
Gender:				
Male	1		1	
Female	1.14 (0.88- 1.47)	0.29	0.96 (0.61-1.51)	0.87
Stage:				
I	1		1	
II	1.23 (0.77-1.95)	0.38	2.02 (1.14-3.55)	0.02
III	1.93 (1.24-3.00)	0.00	2.15 (1.24-3.74)	0.01
IV	2.70 (1.80-4.06)	0.00	3.53 (2.09-5.94)	0.00
Unknown	1.15 (0.63-2.13)	0.64	2.16 (0.95-4.89)	0.07
Treatment:				
RT	1		1	
Surgery	0.33 (0.18-0.61)	0.00	0.17 (0.02-1.27)	0.09
Surgery+RT	0.62 (0.45-0.84)	0.00	0.57 (0.40-0.82)	0.00
No	1.29 (0.97-1.71)	0.08	2.37 (1.79-3.13)	0.00
Differentiation:				
Well	1		1	
Moderate	0.96 (0.71-1.32)	0.84	0.89 (0.65-1.22)	0.49
Poor	1.68 (1.02-2.79)	0.04	0.75 (0.51-1.09)	0.14
Unknown	0.72 (0.53-0.97)	0.04	0.84 (0.56-1.10)	0.16

* 'HR, hazard ratio; CI, confidence interval

Table 4. Multivariate Cox Regression Analysis of Hypopharynx and Larynx Cancer

Variables	Hypopharynx		Larynx	
	HR (95% CI)	p	HR (95% CI)	p
Age:				
<60	1		1	
60-69	0.83 (0.52-1.22)	0.31	1.27 (0.79-2.05)	0.32
70-79	0.98 (0.65-1.48)	0.94	1.55 (0.94-2.56)	0.08
> 80	1.22 (0.67-2.22	0.51	5.31 (2.63-10.7)	0.00
Gender				
Male	1			
Female	0.96 (0.52-1.73)	0.87	0.65 (0.29-1.45)	0.30
Stage				
I	1			
II	1.14 (0.34-3.81)	0.84	2.74 (1.31-5.74))	0.01
III	1.55 (0.59-4.12)	0.38	5.15 (2.68--9.90)	0
IV	3.23 (1.29-8.12)	0.01	8.47 (4.53-15.8)	0
Unknown	3.90 (1.29-11.8)	0.02	5.29 (2.14-13.1)	0
Treatment				
RT	1			
Surgery	2.46 (0.57-10.6)	0.23	1.07 (0.32-3.59)	0.90
Surgery+RT	0.68 (0.43-1.07)	0.10	0.49 (0.31-0.81)	0.01
No	1.93 (1.28-2.91)	0.00	1.98 (1.27-3.10)	0.00
Differentiation				
Well	1			
Moderate	1.01 (0.64-1.59)	0.96	1.60 (0.98-2.62)	0.06
Poor	0.82 (0.45-1.49)	0.51	0.78 (0.40-1.49)	0.45
Unknown	1.23 (0.79-1.96)	0.34	0.95 (0.59-1.51)	0.82

associated with survival for all the four sites, while age was significant in the larynx and grade was only significant in hypopharyx cancer.

In multivariable analysis (Table 3 & 4), the results were consistent with the univariate analysis. Stage and treatment were strong prognostic factors for 5-year OS in all sites. An age > 80 years are significantly associated with poor survival in oral cavity and larynx cancer. For the oropharynx, hypopharynx and larynx, an unknown stage was associated with poor survival which is similar to stage III/IV; whereas, in oral cavity cancer, it did not differ from stage I/II. Regarding treatment type, surgery was associated with the best 5-year OS in oral cavity and oropharyx cancer, but with very poor survival in hypopharynx and larynx cancer. The two patients with hypopharyx cancer who received surgical treatment (total laryngectomy) had stage III and IV diseases and one of them died from postoperative sepsis. For the larynx, three of the six patients treated with surgery had stage I and the other three had advanced stages or unknown stage.

Discussion

Head and neck cancers are diseases associated with high morbidity and mortality. They are prevalent in developing countries including Thailand. In the present study, consecutive new cases of oral cavity, oropharynx, hypopharynx and larynx cancers diagnosed during 2002 to 2004 were analyzed for their 5-year overall survival and associated clinicopathological variables. The results reveal very low 5-year overall survival rates which are significantly related to the advanced stages at presentation and the treatment modality used.

The present study revealed considerably low 5-year survival rates in all the four anatomical sites of HNCA. Cancer of the larynx had the best survival rate (38%) followed by oral cavity (25.91%), oropharynx (19.24%) and hypopharynx (13.24%). This trend of ordering is similar to other reports (Le Tourneau et al., 2005). The 5-year survival rates in the present study are notably lower than other reports, especially those from Western countries (Carvalho et al., 2005; Barzan et al., 2002; MacKenzie et al, 2009). The analyses of the SEER database in the US during 1992 to 1997 revealed 5-year overall survival rates for oral cavity/pharynx cancer of 56.3% and for larynx of 63.5%, which are nearly double our figures.3 In addition, the authors analyzed the time trends over twenty years and found a notably increased survival rate in oropharynx cancer (36.3% to 49.1%, p = 0.001) and hypopharyx cancer (28.3% to 33.3%; p = 0.015). The increase in survival rates during the years for these cancers is thought to be due to the increased combined surgery and radiation modality (21% to 34%). The vast improvement in the survival rate of oropharynx cancer patients from the increase in combined surgery and radiation rather than radiation alone is also reported in European countries (Mäkitie et al., 2009; Lybak et al., 2011). The smaller proportion of patients receiving this combined treatment could be one of the reasons contributing the poor survival in our series.

In the present study, clinical stage was found to be the strongest prognostic factor for survival which is consistent with most other studies (Pericot et al., 2000; Yeole et al., 2003; Rusthoven et al., 2008; De Paula et al., 2009). The advanced stages accounted for 66% of the whole series. This frequency would reach 70%, since most of the patients with an unknown stage (5.7%) were those with advanced diseases who refused treatment or who were absent for treatment after planning were included. However, the proportion of advanced stage at presentation should be lower in the general population because this study was done in a referral university hospital. A considerable high proportion of advanced disease (up to 80%) is also reported in India (Mohanti et al., 2007) and Brazil (De Paula et al., 2009), in contrast with lower stage at presentation in Western countries (Rusthoven et al., 2008; MacKenzie et al., 2009). For example, the SEER data from the Unites States reported advanced stage of oral cavity accounts for 46.7% compared to 59.9% in our series (Carvalho et al., 2005).

Comparing the survival among the four anatomical sites, the larynx and oral cavity have a higher proportion of early disease (36% and 33%, respectively), while hypopharynx had the highest proportion of advanced disease at the time of diagnosis (84.34%). This accounts for the superior survival rates for oral and larynx cancer and the worst survival rate for hypopharynx cancer. Multiple factors may also contribute to advanced stage at presentation, including personal factors, health education, health care access, or others. The previous study from our hospital has revealed that having herbal medicine before seeking professional health care provider is significantly associated with advanced stage at presentation in oral cancer patients (Kerdpon and Sriplung, 2001). Delayed

seeking of care by physicians allows cancer to progress, resulting in advanced disease at presentation.

In HNCA, extent of disease determine the treatment options. Single modality (surgery or radiotherapy) is used for stage I and early stage II cancer and combined surgery and radiotherapy is treatment option for locally advanced tumor. Our results revealed that treatment type strongly influences the survival. In our cohort, the proportion of surgery alone (1.01-8.8%) is remarkably low compared to other series like the series of SEER of the US (10.2-48.9%). This can be expected given the small proportion of localized stage at presentation in our patients. In a report from Northeastern Italy, even with a similar stage distribution to us, a very high proportion of oral cancer (45.6%) and oropharynx cancer (21.5%) received surgery alone compared to 8.78% and 1.40%, respectively, in our series (Barzan et al., 2002). Also, the proportion of patients treated with surgery plus radiation is lower than the aforementioned series stated (Carvalho et al., 2005; Barzan et al., 2002). This combined treatment has been shown to improve locoregional control and overall survival for locally advanced HNCA (Mäkitie et al., 2006; Lybak et al., 2011). This indicates that our patients did not receive the treatment option that should be received according to their stage of disease.

For HNCA, the selection of treatment for each individual depends on various factors, primarily based on the extension of the tumor and the patient's surgical risk. For our patients, their input or decision is also an important determining factor for treatment selection. This is demonstrated by the large number of patients, nearly one-third of the them (330 from a total 1186 cases), who did not proceed for treatment as planned. These patients were slightly older than those who received treatment (68.1 versus 64.4 years). Even though a large proportion of them had stage IV disease (42%), the rest were stage I-III, treatable and would have fully benefitted from treatment. However, the reasons for refusing or their absence for treatment are not known. Determining these reasons would be worthwhile for improving the management of a patient's decision process.

Our results show that older patients were significantly associated with poor survival. This is consistent with other reports (Singh et al., 2000; Warnakulasuriya et al., 2007). However, a few authors have not found the independent effect of age on survival (De Paula et al., 2009). Different cutoff values of age used in these analyses likely effects the results. When we used a cutoff value of younger or older than sixty-five years of age, a significant effect for age on survival was seen only in larynx cancer, not other sites. When we used seventy years as a cutoff value, the significance of age was evident in oropharynx and larynx cancer. Finally, when classifying old age into more than one group (less than 60, 60 to 70; 70 to 80, and greater than 80 years), the distinctive effect of older age on increasing trends was seen. In the study of De Paula et al. (2009), the authors focused on a very young age group (less than 45 years). By using young age as a cut point, the effect of very old age - the sixth, seventh or more decades - may not be seen. The poor outcome in very old patients is known to be related to co-morbidity and treatment related morbidity (Chen et al., 2001; Clark et al., 2006).

In summary, the present data reveals the unfavorable outcomes of head and neck cancer patients in our population. Patients came at an advanced stage of disease which critically effects treatment results and prognosis. Therefore, efforts to increase the proportion of patients with early stage cancer is a major concern. Also, treatment efficiency should be improved, in particular, combined treatment modality. Finally, effective patient education and communication are serious concerns for maximizing the number of patients achieving treatment as planned.

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Brief communication (Original)

Prognostic significance of p16, p53, Bcl-2, and Bax in oral and oropharyngeal squamous cell carcinoma

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Background: The proteins p16, p53, Bcl-2, and Bax are important cell cycle and apoptotic regulators involved in carcinogenesis and found to have prognostic significance in various cancers. However, the data for squamous cell carcinoma of oral cavity (OSCC) and of oropharynx (OPSCC) are conflicting.
Objective: We sought to determine if expression of p16, p53, Bcl-2, and Bax expression are associated with 5-year overall survival (OS) of patients with OSCC and OPSCC.
Methods: One-hundred thirty-seven cases of OSCC and 140 cases of OPSCC diagnosed from January 2002 to December 2004 at Songklanagrind Hospital, Songkhla, Thailand, were analyzed using a Cox proportional hazards model for 5-year OS in relation to immunohistochemical detection of Bcl-2, Bax, p53, and p16 proteins.
Results: The frequencies of p16, p53, Bcl-2, and Bax expression in OSCC were 13%, 45%, 4%, and 66%, and in OPSCC were 18%, 53%, 22%, and 75%, respectively. In univariate analysis, clinical variables including T stage, N stage and treatment were significantly associated with survival. In multivariate Cox regression, Bax overexpression was significantly associated with poor survival both in OSCC (HR 1.77, 95% CI 1.04–3.01) and in OPSCC (HR 2.21, 95% CI 1.00–4.85). We found no significant association of p16, Bcl-2, and p53 expression with survival.
Conclusion: The expression pattern of p16, p53, Bcl-2, and Bax are similar in OSCC and OPSCC. Only Bax expression has prognostic significance for both tumor sites.

Keywords: Bcl-2, Bax, immunohistochemistry, oral cancer, oropharynx, p53, p16, prognostic marker, squamous cell carcinoma

Oral cancer is an important health problem worldwide with an estimated 263,900 new cases and 128,000 deaths occurring in 2008 [1]. Oral cancer represents the twelfth and eighth most common type of cancer in developed and less-developed areas with an age-standardized incidence rate (ASR) of 6.9 and 4.6 per 100,000 males in 2008. It is also common in Thailand with an ASR of 8.3 per 100,000 males in Songkhla Province in southern Thailand [2]. Oropharynx cancer is less common, but it has a higher case-fatality rate than oral cancer. The survival outcome of oral and oropharynx cancers have only subtly increased during the past two decades, by contrast with the advances in their treatment [3]. Identification of biological factors to predict a patient's clinical outcome in planning effective therapeutic strategies is valuable for improvement of patient care.

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Apoptosis and cell cycle control are two intimately linked molecular pathways involved in carcinogenesis and progression of cancer. The anti-apoptotic Bcl-2 and pro-apoptotic Bax are important members of the Bcl-2 family of proteins that play a role in the regulation of apoptosis [4]. p53, a product of *p53* tumor suppressor gene, plays a role both in cell cycle control and apoptosis by inducing growth arrest and initiating apoptosis after exposure to DNA damage [5]. p53 regulates apoptosis via transcriptional activation of Bax and suppression of Bcl-2 [6]. p16 is a cell cycle blocker. It acts by inhibiting cyclin-D1-CDK4/6 complexes that drive G1-S transition of cell cycle [7].

Oral cavity squamous cell carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC) are known to share common etiologic factors including smoking and drinking alcohol. However, recent evidence denotes a substantial proportion of OPSCC being related to human papillomavirus (HPV) infection, which may result in different tumor characteristics and behavior [8, 9]. In this study, we assessed the

expression of the four proteins, Bcl-2, Bax, p53, and p16 by immunohistochemistry and separately evaluated their relationships to survival outcomes in OSCC and OPSCC.

Material and methods

The studied subjects included patients with histologically-proven primary OSCC and OPSCC who sought treatment at Songklanagarind Hospital from January 2002 to December 2004. Case findings and clinical data were collected from patient records in the Department of Otolaryngology Head and Neck Surgery, Faculty of Medicine, Prince of Songkla University. Follow-up information was obtained from this department and from the Cancer Registry Unit of the faculty. The study was reviewed and approved by our Institutional Ethics Committee.

Primary tumors, lymph node involvement, and stage determination were classified according to the International Union Against Cancer (UICC) classification, fifth Edition, 1997. Pretreatment evaluation included routine laboratory testing, chest X-ray and CT scan of head and neck region.

Mortality information was retrieved from the Department of Provincial Administration, Ministry of Interior. Census registration data is linked nationwide and can be assessed with authorized permission. The Cancer Registry Unit of the faculty updates the mortality information from the census registration data twice yearly. Patients not found dead in this database up to December 2008 were designated as being alive.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections. Antigen retrieval was accomplished by immersing slides in Tris-EDTA buffer pH 9 in a pressure cooker at 95°C for 4 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. The sections were incubated with primary antibodies against p53 (clone DO7, DakoCytomation; dilution 1:100), Bcl-2 (clone bcl-2/100/D5, Novocastra; dilution 1:80), Bax (polyclonal, DakoCytomation; dilution 1:150), and p16 (CINtec $p16^{INK4a}$ Histology Kit, DakoCytomation). The sections were then incubated with EnVision for 30 minutes. The slides were incubated with DAB for color development and counterstained with hematoxylin. Sections of esophageal squamous cell carcinoma that are known to be strongly positive for p53 expression and p16 expression were used as positive controls. Sections of endometrial hyperplasia were used as positive controls for Bcl-2 and Bax. In addition, infiltrating lymphocytes were used as internal positive controls for Bcl-2 and Bax expression.

Immunohistochemical evaluation was performed by one pathologist who was blinded to the clinical status and outcome of the patients. Immunoreactivity of Bax and Bcl-2 were observed in the cytoplasm, p53 expression in the nucleus and p16 expression in nucleus and cytoplasm. The percentage of positively stained tumor cells was estimated overall by assessing the whole slide. Staining equal to or less than 5% of Bax, Bcl-2, p16, and 10% of p53 was considered negative. Intensity of staining was assessed as negative, weak, modest, or intense.

Statistical analyses

Statistical analyses were conducted using the STATA statistical software package, version 6.0. Five-year overall survival (OS) of each category of variables was obtained using the Kaplan–Meier method and compared using a log–rank test. The starting date for the analysis was set at the date of definite clinical diagnosis, usually confirmed by pathological reports. The endpoint was the date of death up to December 2008. Cox proportional hazards regression was performed to obtain independent prognostic factors for survival. Protein expression categorized as ≤25%, 26%–50%, and >50% were used in Cox regression. The 5% level of significance was used for all statistical tests. When no significant difference between using percentage and intensity of expression was found, the percentage of expression was used in all analyses.

Results

Patients characteristics

One-hundred thirty-seven cases of OSCC and 140 cases of OPSCC were included for the analysis. The patients' characteristics are shown in **Table 1**. The proportion of cases in women was much greater for OSCC, whereas less-differentiated tumors were more frequently found in OPSCC. The majority of the patients were treated using radiation either with or without surgery. Approximately one-third of the patients were not treated because they refused treatment or did not show for their hospital visit after treatment planning.

Table 1. Clinicopathological features of the patients

Variables	Number of cases (%)	
	Oral cavity	Oropharynx
Age(y) mean (range)	65.3 (30–90)	64.9 (33–89)
Sex		
Male	85 (62)	136 (97)
Female	52 (38)	4 (3)
Stage		
I	17 (13)	10 (7)
II	24 (18)	27 (19)
III	28 (21)	37 (27)
IV	67 (49)	65 (47)
Treatment		
Radiation	37 (27)	66 (47)
Surgery	10 (7)	2 (1)
Radiation and surgery	53 (39)	27 (19)
Chemoradiation	1 (1)	7 (5)
Untreated	36 (26)	38 (27)
Differentiation		
Well	97 (71)	49 (35)
Moderate	30 (22)	65 (46)
Poor	10 (7)	26 (19)

Protein expressions in relation to clinicopathological parameters

The frequencies of p16, p53, Bcl-2, and Bax expression in OSCC were 13%, 45%, 4%, and 66% and in and OPSCC were 18%, 53%, 22%, and 75%, respectively. **Figure 1** shows immunochemical images of representative samples of the four proteins.

The correlations between protein expression and clinicopathological variables are presented in **Tables 2 and 3**. In OSCC, Bcl-2 expression was observed only in moderately-differentiated tumors. In OPSCC, p53 was inversely correlated with degree of differentiation.

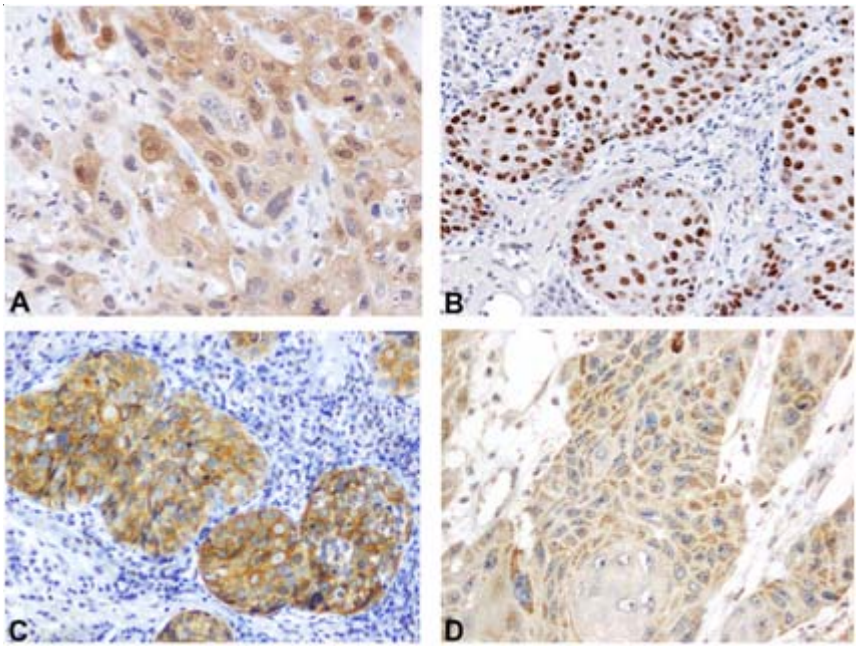

Figure 1. Representative examples of p16 expression (**A**), p53 (**B**), Bcl-2 (**C**), and Bax (**D**). Light microscope magnification 400x (A, C, D) and 200x (B).

Table 2. Clinicopathological variables in relation to protein expression in OSCC.

Variables	Percentage of cases with positive expression, [a]*P*							
	Bcl-2	***P***	**Bax**	***P***	**p53**	***P***	**p16**	***P***
Age, years								
<60	8	0.08	76	0.11	51	0.33	5	0.08
≥60	15		62		42		17	
Sex								
Male	5	0.39	65	0.89	65	0.89	13	0.70
Female	2		67		67		15	
T stage								
T1	4	0.94	48	0.09	57	0.45	18	0.69
T2	3		65		41		15	
T3–T4	4		72		43		11	
N stage								
N0	5	0.58	59	0.05	40	0.14	13	0.93
N1	4		85		62		16	
N2–N3	0		68		41		14	
Differentiation								
Good	0	0	65	0.87	38	0.03	14	0.95
Moderate	17		69		64		13	
Poor	0		70		60		10	

[a]Chi-squared test

Table 3. Clinicopathological variables in relation to protein expression in OPSCC

Variables	Percentage of cases with positive expression, [a]*P*							
	Bcl-2	***P***	**Bax**	***P***	**p53**	***P***	**p16**	***P***
Age, years								
<60	12	0.03	82	0.17	53	0.93	17	0.84
≥60	28		72		52		18	
Sex								
Male	22	0.88	75	0.25	75	0.25	18	0.71
Female	25		100		100		25	
T stage								
T1	37	0.11	93	0.07	58	0.86	19	0.45
T2	18		71		51		23	
T3–T4	18		70		52		14	
N stage								
N0	17	0.60	69	0.52	58	0.50	20	0.14
N1	25		78		52		6	
N2–N3	24		78		47		22	
Differentiation								
Good	23	0.07	69	0.41	57	0.13	7	0.01
Moderate	23		78		56		19	
Poor	36		81		34		35	

[a]Chi-squared test

Clinical variables and protein expression in relation to survival

Median survival time of the patients with OSCC was 13.2 months (range 0.13–84.7 months). The median survival time of OPSCC was 10.8 months (range 0.9–77.2 months). The 5-year OS rate of OSCC was 23% (95% CI, 16.7–30.8) and of OPSCC of 16% (95% CI, 10.3–23.0). The results of analyses in both OSCC and OPSCC are similar. In univariate analysis, only clinical parameters including T stage, N stage, and treatment, but none of the protein expression showed significant association with survival by log–rank tests (data not shown). In multivariate Cox regression (**Table 4**), however, Bax expression appeared to be significantly associated with poor survival. p16 expression tended to be related with favorable prognosis, but this was not significant. Bcl-2 and p53 expression also showed no significant association with survival.

Discussion

Evidence published during the last decade denotes a substantial proportion of HNSCC (about 20%), in particular in the oropharyngeal site (up to 45%) is HPV-induced, which is related to a more favorable prognosis compared with non-HPV-related tumor [10]. High-risk HPV oncoproteins disrupt the functional protein complex pRB-E2F, leading to the transcription of *p16* genes that promote cell proliferation. Immunohistochemically identified p16 overexpression is proposed to be a surrogate marker for HPV detection in HPV-related cancer, with a sensitivity and specificity of more than 90% and 80% respectively [11]. Our results showed that the frequency of p16 expression is slightly higher in OPSCC compared with OSCC, which is consistent with its probable link to HPV infection. However, the frequency is remarkably low compared with others [9]. HPV may probably not play an important role in Thai patients as reported

Table 4. Multivariate Cox regression analyses

Variables	Oral cavity			Oropharynx		
	HR	95% CI	*P*	HR	95% CI	*P*
Age ≤60 years	1			1		
Age >60 years	1.07	0.65–1.77	0.77	1.22	0.76–1.96	0.41
Male	1			1		
Female	1.61	0.99–2.61	0.054	1.52	0.35-6.65	0.58
T1	1			1		
T2	1.46	0.69–3.04	0.31	1.22	0.65–2.25	0.53
T3–T4	1.93	0.99–3.76	0.053	0.92	0.50–1.69	0.80
N0	1					
N1	1.23	0.69–2.17	0.48	1.83	1.04–3.24	0.04
N2–N3	1.89	1.04–3.42	0.04	4.15	2.48–6.93	<0.001
Untreated	1			1		
Radiation	0.78	0.45–1.36	0.39	0.28	0.17–0.47	<0.001
Surgery	0.24	0.08–0.75	0.014	N/A	-	-
Radiation and surgery	0.34	0.18–0.62	<0.001	0.18	0.09–0.37	<0.001
Chemoradiation	0.93	0.09–8.87	0.95	0.05	0.01–0.23	<0.001
Good differentiation	1			1		
Moderate differentiation	1.02	0.56–1.87	0.92	1.22	0.71–2.09	0.47
Poor differentiation	0.98	0.47–2.07	0.97	1.05	0.55–2.02	0.87
Bcl-2, ≤25%	1			1		
Bcl-2, 26%–50%	N/A	–	–	0.44	0.09–2.05	0.30
Bcl-2, >50%	1.77	0.34–9.28	0.50	0.64	0.32–1.25	0.19
Bax, ≤25%	1			1		
Bax, 26%–50%	1.58	0.69–3.63	0.28	2.21	1.00–4.85	0.049
Bax, >50%	1.77	1.04–3.01	0.04	1.09	0.66–1.81	0.72
p53, ≤25%	1					
p53, 26%–50%	1.37	0.62–3.01	0.44	1.09	0.54–2.22	0.81
p53, >50%	1.52	0.85–2.71	0.15	0.69	0.44–1.10	0.13
p16, ≤25%	1					
p16, 26%–50%	0.88	0.25–3.06	0.84	0.79	0.32–2.02	0.64
p16, >50%	0.89	0.31–2.58	0.83	0.77	0.39–1.53	0.45

after one study from Thailand where only one case positive for HPV-DNA was found in 32 cases of OSCC [12]. However, the true prevalence of HPV-related OPSCC in Thai patients needs to be confirmed in a larger sample with accurate techniques to clarify this point. Our results showed that p16 expression tended to be associated with a favorable prognosis, but this was not significant. The lack of significance of the low frequency of p16 expression is probably the result of a lack of sufficient power in the current sample size to reach significance.

The prognostic significance of p53 alterations in OSCC/OPSCC is inconsistent [13]. Consistent with some others [14-17], the current study revealed a nonsignificant association of p53 expression with survival. A recent meta-analysis has demonstrated a significant effect of p53 expression on overall survival in oral cancer [13]. However, most of the studies included in that systematic meta-analysis used a small number of subjects, therefore, the results may be subject to a sampling bias. The current study, which included more than one hundred patients, provides strength for our results. Together this evidence indicates a lack of promise for prognostic role for p53 expression in OSCC/OPSCC.

The low frequency of Bcl-2 and high frequency of Bax in the tumor studied is consistent with previous studies [18-20]. Bcl-2 expression is more likely to be seen in less-differentiated tumors, which was also reported after other studies [17, 18]. Consistent with most previous reports of OSCC/OPSCC [17, 20, 21], we found no prognostic significance of Bcl-2 expression in this cancer, although few studies reported significant association [19, 22].

Because Bax promotes apoptosis, Bax overexpression might be expected to be related to a better prognosis; however, only few studies support this [22], whereas others did not [19-21]. Surprisingly, we found high Bax expression (>25% of positive cells) was significantly associated with poorer survival compared with negative/low expression. To our knowledge, this direction of association has not yet been reported in oral cancer, but a similar finding has been reported in breast and rectal cancers [23, 24]. Aside from apoptotic function, some apoptotic proteins also involve in cell cycle control [25]. Bax has been found to play a role in cell proliferation by accelerating S-phase progression [26]. This may explain our finding of an adverse prognostic role for Bax. However, the actual mechanism contributing to this association needs to be explored further.

In conclusion, the present study demonstrates a similar expression profile for p16, p53, and Bax in OSCC and OPSCC. Bax expression was found to be associated with poor survival, which is rarely reported and needs to be explored further.

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