รายงานวิจัยฉบับสมบูรณ์

การศึกษายีน SHH ในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน

Study of SHH gene in ameloblastoma

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สัญญาเลขที่

MRG5380010

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Project Title : Study of SHH gene in ameloblastoma

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Abstract:

Ameloblasoma is an aggressive odontogenic tumor with unknown etiology. SHH is an important gene in sonic hedgehog signaling pathway. We hypothesized that SHH gene is function as tumor suppressor gene in ameloblastoma and aimed to study the mechanism to suppress the expression. Six cases of ameloblastoma were laser captured and performed RTPCR compared to normal tooth bud. Three of six were found under expression. Then, these three cases were test for copy number variation by Human CytoSNP array, promoter methylation by methylation specific PCR and DNA mutation by DNA sequencing. The results showed that, no gain or loss at chromosome 7q36 and no DNA mutation at exon1, 2, 3 of SHH gene were found. Moreover, the promoter methylation of SHH gene was not associated with expression. These data indicated that SHH gene expression in ameloblastoma may control by orther mechanisms rather than copy number variation, promoter methylation and DNA mutation. However, further investigation in the larger ameloblastoma population is needed.

Keywords : Ameloblastoma, SHH gene, Promoter methylation, DNA mutation

รหัสโครงการ: MRG5380010

ชื่อโครงการ: การศึกษายีน SHH ในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน

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บทคัดย่อ:

โรคเนื้องอกเซลล์ต้นกำเนิดฟันเป็นโรคที่พบบริเวณใบหน้าและขากรรไกรที่มีความรุนแรงของโรคสูงและยังไม่ทราบสาเหตุการเกิดโรค ยีน SHH เป็นยีนที่มีความสำคัญใน sonic hedgehog signaling pathway ที่มีความสำคัญต่อขบวนการสร้างฟัน และการเกิดมะเร็งหลายชนิด การศึกษานี้มุ่งเน้นไปที่ความสำคัญของยีน SHH ในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน โดยตั้งสมมติฐานว่า ยีน SHH ทำหน้าที่เป็นยีนต้านมะเร็ง ผลจากการวัดการแสดงออกของยีนโดยวิธี RTPCR พบว่ามีการลดลงของการแสดงออกของยีน 3 ใน 6 ราย จาก 3 รายนี้นำไปศึกษาหากลไกที่ทำให้ยีนมีการแสดงออกลดลงโดยศึกษา การเพิ่มหรือลดลงของยีน โปรโมเตอร์เมทิลเลชั่น และ ดีเอนเอ มิวเตชั่น ผลของการศึกษาแสดงให้เห็นว่าไม่พบความสัมพันธ์ระหว่างกลไกทั้ง 3 ต่อการแสดงออกของยีนที่ลดลง แสดงว่าให้ให้เห็นว่าน่าจะมีกลไกอื่นๆที่ควบคุมการแสดงออกของยีนนี้ในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน การศึกษาเพิ่มเติมในระยะต่อไปควรจะเพิ่มจำนวนตัวอย่างในการศึกษาให้มากขึ้นและศึกษากลไกอื่นๆ ที่ควบคุมการแสดงออกของยีน

คำหลัก: เนื้องอกเซลล์ต้นกำเนิดฟัน, ยีน SHH, โปรโมเตอร์ เมทิลเลชั่น, ดีเอนเอ มิวเตชั่น

สารบัญ

1. บทนำ

โรคเนื้องอกเซลล์ต้นกำเนิดฟัน (ameloblastoma) เป็นโรคเนื้องอกชนิดไม่ร้ายแรงพบได้บ่อยในกระดูกขากรรไกร มีรายงานครั้งแรกตั้งแต่ปี ค.ศ. 1868 โดย Broca [1] ชื่อ ameloblastoma ถูกตั้งโดย Churchill ในปี ค.ศ. 1933 [1-3] เนื้องอกชนิดนี้เป็นเนื้องอกที่พบบ่อยที่สุดในกลุ่มเนื้องอกที่มีต้นกำเนิดมาจากฟัน (odontogenic epithelium) โดยพบประมาณ 13-24% ตามแต่กลุ่มประชากรที่ใช้ศึกษา [4] และ พบได้ประมาณ 1% ของรอยโรคในช่องปาก [5] สำหรับในประเทศไทยจากการศึกษาโดยรัฐพงษ์ วรวงศ์วสุ [6] โดยศึกษาจาก รอยโรคในช่องปากจำนวนใน 2,408 รายในปีพ.ศ. 2516-2540 พบว่ารอยโรคเนื้องอกจากเซลล์ต้นกำเนิดฟันมีอัตราการเกิดมากเป็นลำดับที่ 5 ของรอยโรคทั้งหมดที่พบในช่องปาก และมีอัตราการเกิดโรคในกระดูกขากรรไกร และข้อต่อขากรรไกรมากเป็นลำดับที่ 2 (24.8%, 193 ใน 779 ราย) ตำแหน่งที่เกิดมากกว่าร้อยละ 80 พบที่ขากรรไกรล่าง โดยเฉพาะบริเวณฟันกรามและส่วนท้ายฟันกรามเป็นตำแหน่งที่พบมากที่สุด [7] มักจะพบความชุกสูงในช่วงอายุ 30-50 ปี [8,9]

เซลล์ต้นกำเนิดของเนื้องอกชนิดนี้มีรายงานว่าเกิดจาก [2]

1. เซลล์เยื่อบุที่เหลือค้างของเนื้อเยื่อที่สร้างฟัน ได้แก่ แถบเยื่อบุผิวต้นกำเนิดฟัน (Dental lamina), epithelial rest of Malesses เป็นต้น
2. เซลล์เยื่อบุของ odontogenic cyst
3. Basal cell ของเนื้อเยื่อช่องปาก
4. เซลล์เยื่อบุของ Enamel organ
5. เยื่อช่องปากที่เกิดอยู่นอกช่องปาก

เนื้องอกจากเซลล์ต้นกำเนิดฟันจะมีลักษณะจุลพยาธิวิทยาจะอยู่ในกลุ่มเนื้องอกที่ไม่ร้ายแรง เจริญเติบโตช้า และไม่สัมพันธ์กับอาการแสดงออกจนกว่าจะมีขนาดใหญ่ขึ้น ถึงแม้เนื้องอกชนิดนี้จะเป็นเนื้องอกที่ไม่ร้ายแรงแต่พฤติกรรรมเฉพาะของโรคจะคล้ายมะเร็ง คือมีการทำลายกระดูก ลุกลามเฉพาะที่เข้าสู่เนื้อเยื่อข้างเคียง ขอบเขตของโรคไม่ชัดเจน และมีอัตราการเกิดซ้ำสูง 50-72% (10-12) เนื้องอกที่มีขนาดใหญ่ขึ้นเรื่อยๆจะทำให้ผู้ป่วยเกิดความไม่สบาย ในการดำรงชีวิต [9]

เนื้องอกจากเซลล์ต้นกำเนิดฟัน แบ่งตามลักษณะทางจุลพยาธิวิทยาได้ 4 ชนิด คือ solid, unicystic, peripheral และ desmoplastic [13,14] โดยทุกชนิดมีลักษณะร่วมกันคือ มีการรวมกลุ่มของเนื้องอก ลอยอยู่ใน เนื้อเยื่อยึดต่อที่โตเต็มที่แล้ว (mature fibrous tissue) กลุ่มของเนื้องอกจะพบเยื่อบุที่สานกันไปมาเป็นร่างแห ชั้นล่างเป็นเซลล์ cuboidal หรือ columnar เรียงตัวตั้งฉากกับฐาน (basement membrane) ซึ่งเซลล์ในกลุ่มนี้ก็คือ ameloblast or preameloblast-like cells เซลล์ชั้นในเป็นเซลล์รูป stellate อยู่กันอย่างหลวมๆ บางครั้งจะพบ การเกิดถุงน้ำ (cystic degeneration) ในชั้นด้านใน ซึ่งเกิด

จากการเสื่อมสลายของเซลล์ใน stellate reticulum [15,16] ลักษณะที่แตกต่างกันทางจุลพยาธิวิทยาของทั้ง 4 ชนิด ไม่สัมพันธ์กับลักษณะทางคลินิก, การเกิดเป็นซ้ำ และการพยากรณ์โรค [17]

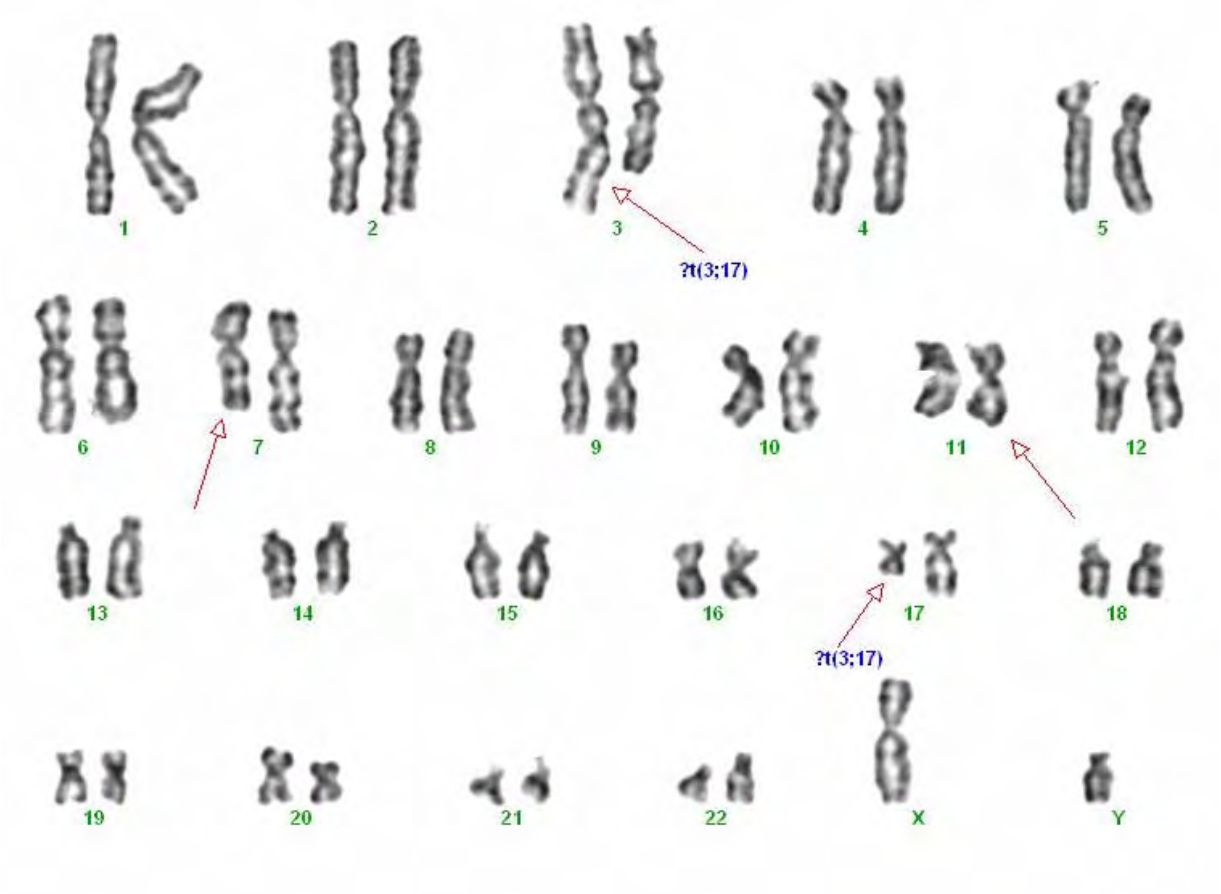
โรคเนื้องอกจากเซลล์ต้นกำเนิดฟันจะไม่ตอบสนองต่อการรักษาโดยวิธีเคมีบำบัด และรังสีบำบัด การรักษาผู้ป่วยจึงจำเป็นที่จะต้องใช้วิธีการทำศัลยกรรมอย่างกว้างโดยวิธีเดียว [18] ลักษณะการทำศัลยกรรมมี 2 แบบ คือการทำศัลยกรรมแบบอนุรักษ์ และ การทำศัลยกรรมแบบ radical อัตราการเกิดเป็นซ้ำของเนื้องอกจะสัมพันธ์กับชนิดของการทำศัลยกรรม [17] โดยถ้ามีการศัลยกรรมแบบ radical อัตราการเป็นซ้ำจะอยู่ระหว่าง 18-27% เปรียบเทียบกับการทำศัลยกรรมแบบอนุรักษ์ อัตราการเป็นซ้ำจะเกิดประมาณ 20-90%

ข้อมูลทางชีวโมเลกุลเพื่อใช้เปรียบเทียบชีววิทยาและพฤติกรรมของโรคยังไม่เป็นที่ทราบ [19] การศึกษาที่ผ่านมาส่วนใหญ่จะเน้นไปที่การศึกษาการแสดงออกของยีนในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน ส่วนใหญ่จะทำโดยใช้วิธีอิมมูโนฮิสโตเคมี (Immunohistochemistry) [13,14, 20-21] ซึ่งเป็นวิธีที่มีความจำกัด

การศึกษาของ Heikinheimo และคณะ [22] โดยการทำ Expression microarray ของเนื้องอกเซลล์ต้นกำเนิดฟัน 8 ราย เทียบกับ หน่อฟันน้ำนม 20 ราย พบว่ายีน *FOS* และ *TNRSF1A* มีการแสดงออกของยีนที่เพิ่มมากขึ้น และยีน *SHH, TRAF3, ARHGAP4, DCC, CDH12, CDH13, TDGF1* และ *TGFB1* มีการแสดงออกลดลง เมื่อเทียบกับเซลล์ต้นกำเนิดฟันในทุกราย อย่างมีนัยสำคัญโดยเฉพาะในยีน *SHH* เมื่อมีการทำ Realtime RT PCR พบว่าเป็นยีนที่มีการแสดงออกลดลงมากที่สุดถึงประมาณ 3 เท่า

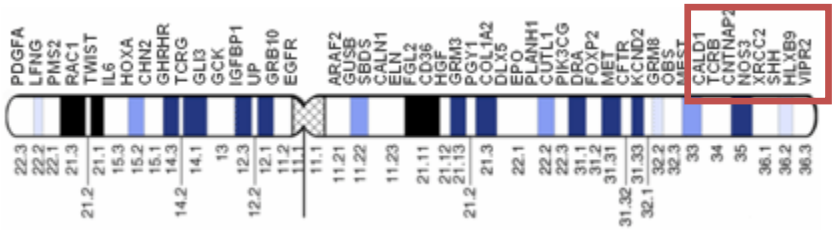
นอกจากนี้ผู้วิจัยได้ทำการทดลองเบื้องต้นโดยการทำ Karyotype จาก primary cell culture ของโรคเนื้องอกเซลล์ต้นกำเนิดฟัน และพบว่า 5 ใน 7 metaphase ที่วิเคราะห์ได้ มี translocation ของ chromosome 3 กับ17 และมี deletion ที่บริเวณ chromosome 7qter และ 3qter ตามรูปที่ 1

รูปที่ 1 Karyotype ของ Ameloblastoma primary cell culture



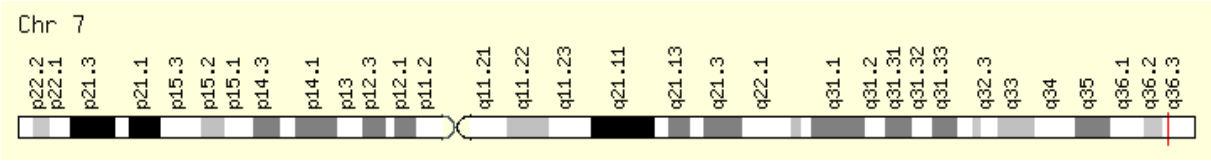
การขาดหายไปบริเวณโครโมโซม 7 ตั้งแต่ประมาณ 7q33-7qter ในบริเวณนั้นเป็นบริเวณที่มียีนต่างๆ ได้แก่ยีน *CALD1, TCRB,CNTNAP2, NOS3, XRCC2, SHH, HLXB9, VIPR2* ดังแสดงในรูปที่2

รูปที่ 2 ยีนสำคัญที่อยู่บนโครโมโซม 7



ยีน Sonic hedgehog *(SHH)* [23-25] เป็นยีนที่อยู่บริเวณโครโมโซม 7q36.3 (รูปที่ 3) ตั้งแต่ 155,288,319 bp จาก pter ถึง155,297,728 bp จาก pter มีขนาด DNA 9,410 bp transcribe ได้ mRNA ขนาด 1,577 bp (3 exons) encode ให้โปรตีน ขนาด 462 amino acids หนัก 49,607 dalton

รูปที่ 3 แสดงตำแหน่งของ SHH บนโครโมโซม 7

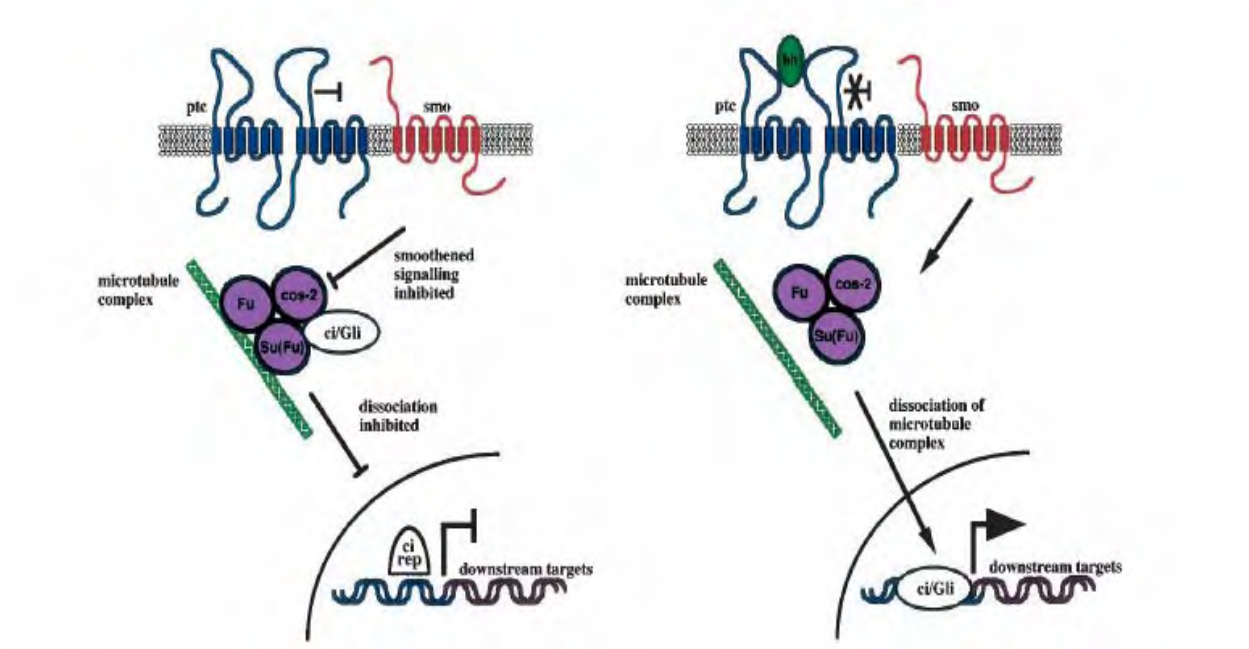


SHH เป็น secretory protein มีหน้าที่เป็นจุดเริ่มต้นของ Hedgehog signaling pathway โดยจะจับและกระตุ้น membrane receptor complex (PTC และ SMO) ยีนที่เป็นต้นทางของ pathway นี้นอกจาก *SHH* จะประกอบไปด้วย *PATCH* เป็นยีนขนาดใหญ่มี 12 transmembrane domain ซึ่งจะ encode ให้โปรตีน PTC และ *Smoothed* มี 7 transmembrane domain ซึ่งจะ encode ให้โปรตีน SMO การกระตุ้น pathway นี้จะทำให้เกิดทำให้เกิดการกระตุ้นโปรตีนต่างๆตามลำดับที่อยู่ downstreamต่อมาตามรูปที่ 4 ทำให้มี cell proliferation และ differentiation อย่างสมดุล ในกรณีที่ไม่มี SHH มาจับ PTC PTC จะยับยั้ง SMO ในช่วง posttranscriptional modification ทำให้ไม่มีการกระตุ้น pathway ในกรณีที่มี SHH มาจับ PTC PTC จะกระตุ้น SMO ทำให้มีการกระตุ้น pathway ซึ่งเป็นผลให้มี cell proliferation และ transcription activation ของยีนต่างๆ เช่น *WNT, BMPs, Gli* เป็นต้น

ความสำคัญของ Hedgehog signaling pathway คือจะควบคุมการ development และ tissue homeostasis ของส่วนต่างๆ ของร่างเช่น แขน,ขา, กระดูกแกน, ปอด, ระบบทางเดินอาหาร, ฟัน และผิวหน้ง ความผิดปกติที่เกิดจากการมีการกระตุ้นมากหรือน้อยกว่าปกติจะทำให้เกิดโรคทางพันธุกรรม และมะเร็งเกิดขึ้นได้เช่น mutation ของ *PATCH* สัมพันธ์กับโรค Nevoid basal cell carcinoma syndrome, *SHH* mutation จะสัมพันธ์กับ Holoprosencephaly ยีนที่อยู่ในกลุ่มของ Hedgehog pathway จัดได้ว่าเป็นยีนต้านมะเร็ง มีรายงานถึงการเกิด mutation ในกลุ่มยีนเหล่านี้ที่พบได้ในมะเร็งชนิดต่างๆ เช่น medulloblastoma, meningioma, breast cancer, esophageal cancer, rhabdomyosarcoma, primary neuroendocrine tumor

ในกระบวนการสร้างฟัน ยีน *SHH* เป็นยีนที่มีการแสดงออกระหว่างการสร้างฟันในหนูทดลอง ถูกสร้างโดย dental epithelium และ ทำให้มีการกระตุ้น Hedgehog signaling pathway[26] นอกจากนี้การที่มีความผิดปกติของการควบคุม pathway นี้ หรือมี mutation ของ PATCH ก็เป็นสาเหตุให้เกิดโรค odontogenic keratocyst[27,28]ซึ่งเป็นเนื้องอกที่พบได้ในช่องปาก มีต้นกำเนิดมาจากเซลล์กลุ่มเดียวกับเซลล์ที่เป็นต้นกำเนิดของเนื้องอกเซลล์ต้นกำเนิดฟัน

รูปที่ 4 Hedgehog signaling pathway แสดงการกระตุ้น ในกรณีที่ไม่มี SHH (ซ้าย) และกรณีที่มี SHH (ขวา)



สมมติฐานที่ทำให้ยีนต้านมะเร็งสูญเสียการทำงาน เป็นไปตาม Knudson's two hit hypothesis โดยที่ จะต้องมีความผิดปกติทั้ง 2 อัลลีล ความผิดปกติครั้งแรกของยีนต้านมะเร็งจะเกิดขึ้นอย่างจำเพาะเจาะจงตรงบริเวณตำแน่งของยีนหรือตำแหน่งของ DNA ที่อยู่ข้างเคียงยีนนั้น ความผิดปกติครั้งแรกนี้อาจเกิดจากความผิดปกติระดับโมเลกุลได้หลายชนิด เช่น point mutation, gene deletion, gene rearrangement ความผิดปกติครั้งที่สองของยีนต้านมะเร็งมักจะเกิดจากความผิดปกติของการแบ่งตัว (mitosis) กลไกที่ทำให้เกิดการสูญเสียหน้าที่ครั้งที่สองอาจเกิดได้จากกลไกต่างๆ ตามรูปที่ 4 [29-30]ดังนี้

1. Non-disjunction and chromosome loss คือมีการขาดหายไปของโครโมโซมที่บรรจุอัลลีลที่ทำหน้าที่ทั้งเส้น

2. Non- disjunction and reduplication คือมีการขาดหายไปของโครโมโซมที่บรรจุอัลลลีลที่ทำหน้าที่ทั้งข้าง และโครโมโซมอีกข้างที่ไม่มีอัลลีลที่ทำหน้าที่มีการแบ่งตัวเพิ่มขึ้นมาแทน

3. Mitotic recombination คือมีการขาดหายไปของชิ้นส่วนของโครโมโซมบริเวณที่มียีนต้านมะเร็งทำหน้าที่บรรจุอยู่และมีชิ้นส่วนของโครโมโซมอีกข้างที่ไม่มีอัลลีลที่ทำหน้าที่เข้ามาแทนที่

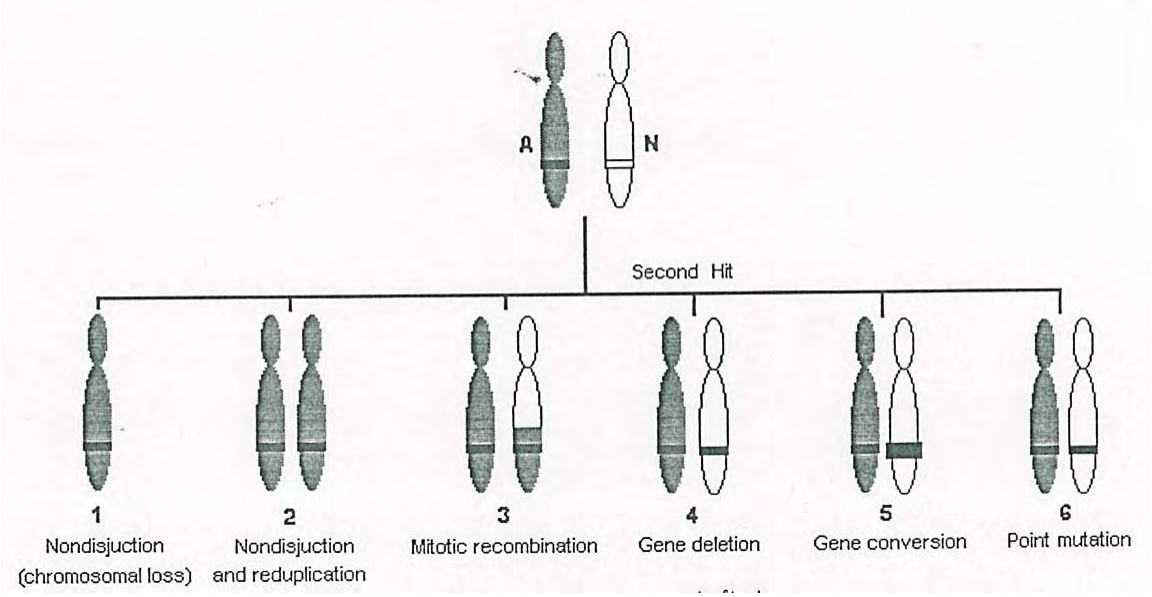
4. Chromosome deletion คือมีการขาดหายไปเฉพาะส่วนของโครโมโซมที่บรรจุอัลลีลที่ทำหน้าที่

5. Gene conversion คือมีการกลับทิศของโครโมโซมในส่วนของโครโมโซมที่บรรจุอัลลีลที่ทำหน้าที่

6. Point mutation ตรงตำแหน่งของยีนต้านมะเร็งมีการผ่าเหล่าเฉพาะจุดเกิดขึ้น

7. กลไกในระดับ epigenetic อื่นๆ เช่น Acetylation, Methylation

รูปที่ 5 แสดงกลไกที่ทำให้เกิดการสูญเสียหน้าที่ครั้งที่สองของยีนต้านมะเร็ง



กลไกต่างๆที่เกิดขึ้นทั้งสองครั้งจะทำให้ยีนต้านมะเร็งไม่ทำงาน จากผลของ cDNA microarray และผล Pilot study ของผู้วิจัยในโรคเนื้องอกเซลล์ต้นกำเนิดฟันจึงนำไปสู่การตั้งสมมติฐานว่าน่าจะมีกลไกที่ทำให้ยีน *SHH* ซึ่งเป็นยีนต้านมะเร็งสูญเสียการทำงาน ผู้วิจัยจึงได้มีความสนใจที่จะศึกษายีน SHH โดยละเอียด โดยจะเน้นไปที่การศึกษากลไกที่ทำให้มีการแสดงออกของยีนลดลง ได้แก่ การศึกษา Promoter methylation, Loss of heterozygosity, DNA mutation ผลที่ได้จากการวิจัยจะทำให้ทราบถึงความสำคัญของยีนSHH และกลไกที่ทำให้มีการแสดงออกของยีนลดลง ซึ่งจะเป็นประโยชน์ในด้านการวินิจฉัยโรค พยากรณ์โรค ตลอดจนเป็นความรู้เบื้องต้นที่จะพัฒนาต่อไปสู่การรักษาในที่สุด

2. วัตถุประสงค์

2.1 เพื่อศึกษาการแสดงออกของยีน SHH ในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน เปรียบเทียบกับเซลล์หน่อฟันปกติ (tooth germ)

2.2 เพื่อศึกษาภาวะ Gain & Loss บริเวณ ยีน SHH บนโครโมโซมคู่ที่ 7 บริเวณ 7q33 ถึง 7q ter ในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน เปรียบเทียบกับเนื้อเหงือกปกติของผู้ป่วย

2.3 เพื่อศึกษาภาวะ Promoter methylation ของยีน SHH ในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน เปรียบเทียบกับเซลล์หน่อฟันปกติ และเปรียบเทียบกับการแสดงออกของยีน

2.4 เพื่อศึกษา DNA mutation ที่ทำให้มีการแสดงออกของยีน SHH ลดลงในโรคเนื้องอกเซลล์ต้นกำเนิดฟัน

3. วิธีการศึกษา

เนื้อเยื่อตัวอย่างที่ใช้ในการทดลอง ผู้วิจัยจะทำการคัดเลือกตัวอย่างเนื้องอกเซลล์ต้นกำเนิดฟันจากผู้ป่วยที่เข้ารับการตรวจรักษาในโรงพยาบาลทันตกรรม คณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล โดยจะเก็บเนื้อเยื่อในรายที่ผู้ป่วยจำเป็นต้องมีการตัดขากรรไกรบางส่วน (partial mandibulectomy) เพื่อการรักษาเท่านั้น เนื้อเยื่อตัวอย่างจะถูกเก็บไว้ใน OCT tissue freezing medium บางส่วนของเนื้องอกจะเก็บในรูปแบบเนื้อเยื่อทางพยาธิ และได้รับการวินิจฉัยโดยพยาธิแพทย์ในคณะผู้วิจัยว่าเป็นเนื้องอกเซลล์ต้นกำเนิดฟัน เนื้อเยื่อบางส่วนจะถูกนำมาเลี้ยงเป็น primary cell culture หรือทำ Laser capture microdissection เพื่อทำให้ได้เซลล์เนื้องอก 100% ไม่มีเซลล์ปกติปนเปื้อน และจะมีการเก็บเนื้อเยื่อบางส่วนจาก flap ของผู้ป่วยในรายเดียวกันมาเป็นเนื้อเยื่อปกติในกลุ่มควบคุมของการทดลองเพื่อหา Loss of heterozygosity

เนื่องจากเนื้องอกชนิดนี้เป็นโรคที่พบได้น้อย จำนวนตัวอย่างที่ใช้ในการทดลองจึงคำนวนตามค่าสถิติของการศึกษาของ Heikinheimo และคณะ โดยผู้วิจัยจะใช้ตัวอย่างเนื้องอกเซลล์ต้นกำเนิดฟันเพื่อทำการวิจัยจำนวน 6 ราย โดยจะมีการเก็บข้อมูลผู้ป่วยจากการทบทวนประวัติการรักษาโดยเก็บข้อมูลทั่วไปของผู้ป่วย ได้แก่ อายุ เพศ อาชีพ ภูมิลำเนา และลักษณะทางจุลพยาธิวิทยาที่สำคัญ สำหรับเนื้อเยื่อกลุ่มควบคุมในการทดลองหา Promoter methylation profile และ gene expression ได้จากหน่อฟันของฟันคุดที่ยังไม่ขึ้นในช่องปากจากผู้ป่วย และจำเป็นต้องผ่าฟันคุดเพื่อการรักษา จำนวน 3 ราย ตัวอย่างเนื้อเยื่อที่ใช้ทำการทดลองทั้งหมดผ่านการเซ็นยินยอมจากผู้ป่วย และผ่านการรับรองจากคณะกรรมการพิจารณาจริยธรรมการวิจัย เนื้อเยื่อที่ถูกคัดแยก นำมาสกัด DNA และ RNA, วัดคุณภาพและปริมาณของ DNA และ RNA ที่ได้ และนำไปทำการทดลองต่อไป

3.1 การศึกษา SHH gene expression โดยการใช้ RNA จาก laser capture microdissection ของ ameloblastoma นำไปตรวจสอบการแสดงออกของยีนเปรียบเทียบกับหน่อฟันปกติ โดยการทำ Real time RTPCR เปรียบเทียบระหว่าง SHH gene กับ GAPDH gene

โดยใช้ primer ตาม protocol ของ Taniguchi และคณะ [31]

SHH RTPCR F: 5'-GTAAGGACAAGTTGAACGCTTTG-3'

SHH RTPCR R: 5'-GATATGTGCCTTGGACTCGTAGTA-3'

3.2 การศึกษา Gain & Loss of gene SHH โดยการทำ HumanCytoSNP microarray (Illumina)[32] การศึกษานี้ใช้ DNA จาก fresh frozen tissue ของ ameloblastoma เปรียบเทียบกับ DNA จากเนื้อ flap คนไข้ในรายเดียวกัน

3.3 การศึกษา Promoter methylation: DNA จากเนื้องอกเซลล์ต้นกำเนิดฟันและจากหน่อฟันปกติในกลุ่มควบคุมจะถูกนำไปทำ sodium bisulfate treatment DNA ที่ได้จะนำไปศึกษา Promoter methytion ต่อไปโดยการทำ Methylation specific PCR (MSP) ตาม protocol ของ Wang และคณะ [33] MSP ใช้ primer ดังนี้

Met specific F: 5' -GGGAGTGGTGGAGAGTTTTTTG-3'

Met specific R: 5' -AACTAATAACTTCCAAACTATCCCCA-3'

Unmet specific F: 5' -GAGCGGTGGAGAGTTTTTCG-3'

Unmet specific R: 5' -ACTAATAACTTCCGAACTATCCCCG-3'

Methylation sequencing ใช้ primer ดังนี้

Met nested F: 5' -GGTGGAGAGTTTTTTGTAGTTGTGGT-3'

Met nested R: 5' -AAACTATCCCCATACAAATCCATACA-3'

Unmet nested F: 5' - AGAGTTTTTCGTAGTCGCGGC-3'

Unmet nested R: 5' - ATCCCCGTACGAATCCGTACG-3'

3.4 การศึกษา DNA mutation โดยการ sequence บริเวณ coding region ของ exon 1, 2, 3 ซึ่งอยู่ใกล้กับตำแหน่ง promoter และมีรายงานการเกิด mutation ตาม protocol ของ Zurawel และคณะ [34]

Exon 1 amplified 2 ส่วน โดยใช้ primer

1-1F: 5'-CCCCCGCGCGCACTCG-3'

1-1R: 5'-TTCCCTTCATACCTTCCGCTG-3'

1-2F: 5'-CCATGTGGCCGAGAAGACCCTA-3'

1-2R: 5'-AAGCAGCGGGTGAAATCACC-3'

Exon 2 amplified 1 ส่วน โดยใช้ primer

2-1F: 5'-TAACGTGTCCGTCGGTGGG-3'

2-1R: 5'-TGCTTTCACCGAGCAGTGG-3'8.

Exon 3 amplified 4 ส่วน โดยใช้ primer

3-1F: 5'-TTCCCTTCTCCTCACCG-3',

3-1R: 5'-CCACAAAGAGCAGGTGCG-3'

3-2F: 5'-TCTTCTACGTGATCGAGACGC-3'

3-2R: 5'-CGGTTGATGAGAATGG-TGC-3'

3-3F: 5'-GTGACCCTAAGCGAGGAGG-3'

3-3R: 5'-CGAGTACCAGTGGATGCCC-3'

3-4F: 5'-AGC-CCTAACGCGTCCAGGTG-3'

3-4R: 5'-TTCAGCTGGACTTGACCGCCAT-3'

ข้อมูลที่ได้จากการทำการทดลองทั้ง 4 การทดลอง จะนำมาวิเคราะห์และสรุป หากลไกที่ทำให้เกิดการแสดงออกของยีน SHH ลดลง

4. ผลการทดลอง

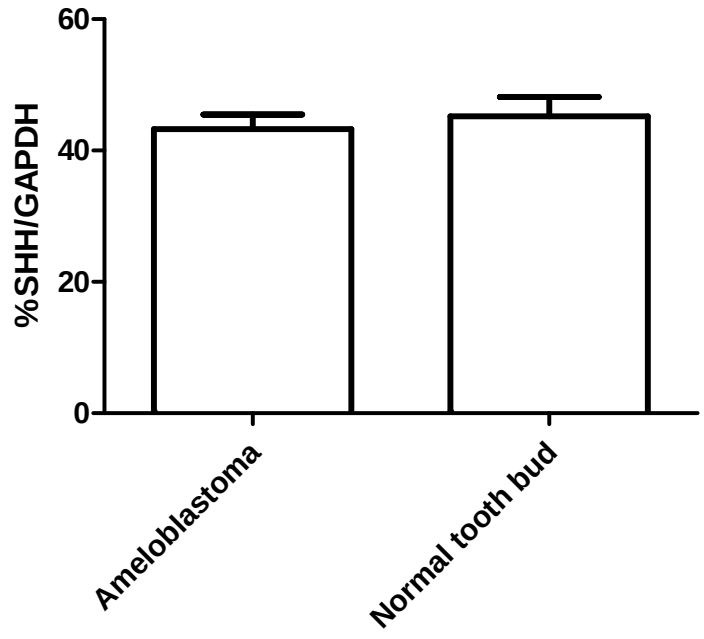
4.1 SHH gene expression

ผลการศึกษาดังแสดงในตารางที่ 1 และ รูปที่ 6

ตารางที่ 1 Percent RTPCR product ของ SHH gene เทียบกับ GAPDH gene

Tissue	Sample	%SHH/GAPDH
Ameloblastoma	A1	50.5
	A2	39.5
	A3	38.3
	A4	46.8
	A5	46.7
	A6	37.8
Normal tooth bud	N1	45.1
	N2	50.4
	N3	40.2

รูปที่ 6 Percent expression ของ SHH gene/GAPDH gene เปรียบเทียบ ระหว่าง ameloblastoma เทียบกับ normal tooth bud



จากการศึกษาพบว่า การแสดงออกของยีนใน ameloblastoma เมื่อเทียบกับ normal tooth bud ไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ (P=0.6166) แต่อย่างไรก็ตาม ใน sample A2, A3 และ A6 มีค่าการแสดงออกของยีนน้อยกว่า normal tooth bud ดังนั้นจึงมีความเป็นไปได้ว่าใน 3 รายนี้จะมีการแสดงออกของยีนที่เหมือนกับยีนต้านมะเร็ง ดังนั้นจึงเลือก sample 3 รายนี้ไปทำการศึกษากลไกต่อ

4.2 SHH gene copy number variation

จากการทำ Human CytoSNP microarray ทั้ง 3 รายได้ผลดังนี้

A2: ไม่พบว่ามีการเพิ่มขึ้นหรือลดลงของchromosome เมื่อเทียบกับเซลล์ปกติ

A3: พบมีการเพิ่มขึ้นของ chromosome 1p33-p32.3

chromosome 2 q32.1

chromosome 22 q12.1-q12.2

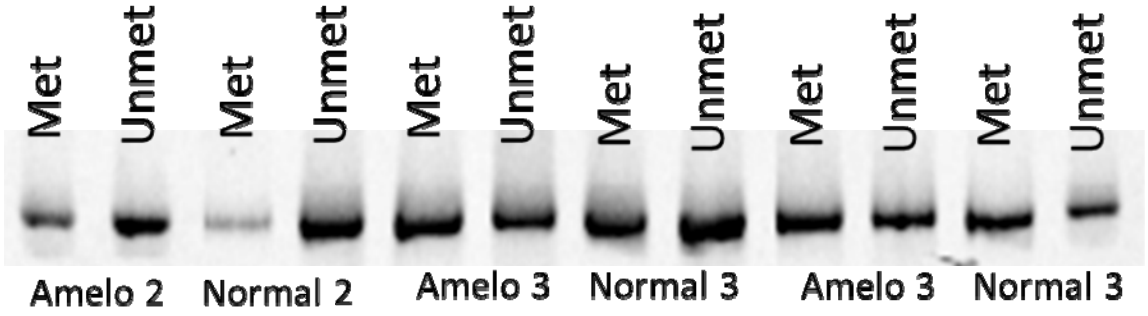
A6: พบมีการเพิ่มขึ้นของ chromosome 15 q13.3

จากผลการทดลอง พบว่าไม่มีการลดลงของ chromosome บริเวณ 7q36 ซึ่งเป็นที่อยู่ของ SHH gene แสดงให้เห็นว่าการเกิด deletion ไม่มีผลต่อการแสดงของ gene SHH ใน ameloblastoma

4.3 Promoter methylation of SHH gene

ผลการศึกษา promoter methylation ดังแสดงในรูปที่ 7

รูปที่ 7 Gel electrophorhesis ของ Promoter methylation เปรียบเทียบระหว่าง Ameloblastoma และ เนื้อเหงือกปกติ (normal) ในผู้ป่วย 3 ราย, Met=methylation, Unmet=unmethylation



จากผลการทดลองพบว่า ใน ameloblastoma และเนื้อเหงือกปกติ พบได้ทั้งภาวะที่มี methylation และ unmethylation และอัตราส่วนของ methylated band ต่อ unmethylated band ก็ไม่ได้มีความแตกต่างกันอย่างมีนัยสำคัญ แสดงให้เห็นว่าใน samples ทั้ง 3 รายนี้ Promoter methylation ไม่ได้มีผลต่อการแสดงออกของ SHH ยีน

สำหรับผลของการ sequencing ผู้วิจัยเลือก sequence ประมาณ 5 clone ต่อ PCR product ผลที่ได้พบทั้ง methylation, unmethylation และ partial methylation ในลักษณะที่ปนกัน แต่ไม่มีความแตกต่างกันระหว่าง ameloblastoma และเนื้อเหงือกปกติ อย่างมีนัยสำคัญ

4.4 DNA mutation of SHH gene

จากการ ทำ PCR, DNA cloning และ DNA sequencing ใน sample ทั้ง 3 ราย ไม่พบว่ามี DNA mutation เกิดขึ้นใน SHH gene บริเวณ exon 1, 2, 3 แสดงให้เห็นว่า DNA mutation ไม่ได้ส่งผลต่อการแสดงออกของยีน

5. สรุปและวิจารณ์ผลการทดลอง

จากผลการทดลองที่ได้ สรุปได้ตาม flow chart ดังนี้

Laser capture microdissect ameloblastoma tissue, normal tooth bud

↓

RT PCR SHH gene 6 ราย

↓

Gene expression ลดลง 3 ราย

↓

Human CytoSNP microarray 3 ราย → no gain or loss at Chromosome 7q36

↓

SHH gene promoter methylation → no association with gene expression

↓

SHH gene mutation → no mutation

ผลการทดลองที่ได้เป็น Negative results ซึ่งต่างจาก การศึกษาของ Heikinheimo และคณะ [22] ซึ่งอาจจะเกิดจากหลายสาเหตุได้แก่ Ameloblastoma เป็นโรคที่พบได้น้อย การเก็บ sample ให้ได้ครบทั้ง RNA, DNA และเนื้อเยื่อปกติทำได้ยากมาก ผู้วิจัยใช้เวลารวบรวม sample ถึง 3 ปี ได้จำนวน sample มาทำการทดลองครบเพียง 6 ราย ดังนั้นถ้าหากมีการเพิ่มจำนวน sample ในการทดลองให้มากกว่านี้ น่าจะทำให้ได้ผลการทดลองที่มีความถูกต้องมากขึ้น

ในการทำ pilot study พบว่า karyotype ของ ameloblastoma ที่ได้มี deletion ที่บริเวณ chromosome 7qter แต่ในการทดลองทำ Human CytoSNP microarray ไม่พบว่ามีการ deletion ที่บริเวณ chromosome 7qter ทั้งนี้เป็นเพราะไม่ใช่ sample เดียวกัน ในการทำการทดลองทั้ง 2 วิธี การพบ chromosome 7qter deletion อาจเป็น sporadic case

การที่พบ sample ที่มีการลดลงของการแสดงออกของยีน SHH เพียง 3 รายเป็นจำนวน sample ที่น้อยทำให้เป็นอุปสรรคในการทำการทดลองต่อในส่วนที่เหลือ ส่งผลให้ผู้วิจัยไม่พบการความเกี่ยวข้องระหว่าง gene expression กับ promoter methylation, copy number variation และ DNA mutation ซึ่งเป็นไปได้ว่า การcontrol gene SHH อาจขึ้นกับกลไดอย่างอื่นเช่น Histone deacethylation, Si RNA, Mi RNA, etc [35]

SHH gene เป็น gene ที่มีความสำคัญใน Hedgehog signaling pathway ซึ่งมีความสำคัญต่อขบวนการ development และมีความสำคัญต่อขบวนการการเกิดมะเร็งชนิดต่างๆ การศึกษา SHH gene ในแง่ของการเป็น tumor suppressor gene ก็มีความสำคัญต่อการพัฒนาไปสู่การรักษาและป้องกันโรคมะเร็งในอนาคต โดยไม่ใช้การผ่าตัด ในกรณี ameloblastoma ผู้วิจัยเสนอให้มีการศึกษาเพิ่มเติมเช่นใน animal model เพื่อความสะดวกในการทดลองมากขึ้น

เนื่องจาการทดลองนี้ได้ผลการวิจัยเป็น negative results ผู้วิจัยได้รับคำแนะนำจากอาจารย์ที่ปรึกษาให้แบ่งทุนวิจัยไปใช้ศึกษาเรื่องอื่นที่มีความเป็นไปได้ในการทำวิจัยมากกว่า ผู้วิจัยจึงแบ่งเงินทุนบางส่วนไปใช้ศึกษามะเร็งชนิดอื่นด้วย ได้แก่การศึกษา HPV physical status และ CCNA1 promoter methylation, ศึกษาระดับ methylation ในเลือดของผู้ป่วยมะเร็งปอด, มะเร็งท่อน้ำดี, มะเร็งตับ, มะเร็งเต้านม, มะเร็งลำไส้ใหญ่ และมะเร็งโพรงหลังจมูก และเขียนบทความปริทัศน์เกี่ยวกับ LINE-1 hypomethylation in cancer ด้วย

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ภาคผนวก

Human Papillomavirus's Physical State and *Cyclin A1* Promoter Methylation in Cervical Cancer

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Background: Cervical cancer is the second biggest cause of death among human female cancers. Human papillomavirus (HPV) is the main factor in this cancer, especially HPV types 16 and 18, which constitute the high-risk group. There are 2 physical states of HPV in host cells: integrated and episomal forms. Our previous study explored the very high degree of *cyclin A1* (*CCNA1*) promoter methylation in invasive cervical cancer in which all cases were infected by HPV.

Objective: From previous evidence, it seemed that HPV might affect *CCNA1* promoter methylation. Therefore, both the quantity and physical state of HPV were investigated in this study for their effects on *CCNA1* promoter methylation.

Materials and Methods: To determine the correlation of HPV quantity and *CCNA1* methylation, the proportion of HPV L1/HAT (histone acetyltransferase, which is a human housekeeping gene) and the percentage intensity of *CCNA1* promoter methylation were observed. *CCNA1* promoter methylation was detected by methylation-specific primer polymerase chain reaction. To investigate the physical state, the HPV *E2* region was amplified. The effect of the physical state on *CCNA1* methylation was observed.

Results: No correlation was found between the quantity of HPV and *CCNA1* promoter methylation. Interestingly, the physical state of HPV had the potential to affect methylation of this gene. The integrated form of HPV had a significantly higher impact on *CCNA1* methylation than HPV in episomal form ($P = 0.001$; 95% confidence interval, 11.96–38.44).

Conclusion: We suggest that the integrated form of HPV might lead to *CCNA1* promoter methylation in cervical cancer by some mechanisms.

Key Words: HPV, Episome, Integrated, *CCNA1* promoter methylation, Cervical cancer

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Cervical cancer is one of the leading causes of death among women. Nearly 100% of cervical cancers are caused by the human papillomavirus (HPV). Human papillomavirus types 16 and 18 constitute the high-risk group.[1] Human papillomavirus type 16 is commonly found in cervical squamous cell carcinoma, whereas HPV 18 is the most prevalent type in cervical adenocarcinoma.[2] The HPV genome consists of 8 genes: *E6*, *E7*, *E1*, *E2*, *E5*, *E4*, *L1*, and

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L2.[3] There are 2 physical states of HPV in host cells: integrated and episomal forms. Integrated HPV is the dominant form of infection. When HPV is in integrated form, the *E2* gene, which suppresses the *E6* and *E7* genes, is disrupted. Disruption of the *E2* gene leads to overexpression of *E6* and *E7*, which are oncogenic genes, eventually causing the transformation of normal cells to cancer cells.[4] Although integrated HPV is the major form of infection, which can lead to carcinogenesis, some studies have shown that the episomal form also leads to cervical cancer.[5,6] Another study has shown that episomal DNA can allow an increase of E6 and E7 by deletion in the YY1 binding site of the long control region of HPV episomal DNA.[7] Thus, both physical states of HPV in a host cell can be the cause of tumorigenesis. In this regard, the absolute levels of HPV can impact this process. For instance, copy number of HPV type 16 has been shown to be significantly associated with tumor initiation[8] and increasing levels corresponding to progression. Furthermore, studies also suggest a positive correlation between E6/E7 messenger RNA level and viral DNA load in invasive cervical carcinoma.[9] Not only HPV causes cervical cancer; loss of function of tumor suppressor genes (TSGs) can also lead to tumorigenesis. There are several factors that evidently lead to dysfunction of these genes. Loss of heterozygosity[10] and promoter methylation of TSGs[11,12] are the main causes of this event in many cancers. In cervical cancer, promoter methylation of TSGs is a risk factor as well.[13,14]

Cyclin A1 (*CCNA1*) is one of the TSGs in many cancers, such as nasopharyngeal carcinoma,[15] head and neck squamous cell carcinoma (HNSCC),[16] and cervical cancer.[13,14] In HNSCC, expression of *CCNA1* has been shown to correlate with TP53 activation. *Cyclin A1* promoter methylation has also demonstrated inverse correlation with *p53* mutation status.[16] The mechanism of loss of function of *TP53* in cervical cancer is different from that in HNSCC. In HNSCC, it is disrupted by gene mutation, whereas in cervical cancer, HPV infection is the main risk factor.[17]

Our previous study found a high degree (93.3%) of *CCNA1* promoter methylation in invasive cervical cancer, correlating with its expression.[14] All cervical cancer cases tested positive for HPV. This evidence prompted us to determine the correlation between HPV and *CCNA1* promoter methylation. In this study, the quantity and physical state of HPV were observed for their impact on *CCNA1* promoter methylation. This correlation is of great interest because it may be evidence that HPV induces promoter methylation of TSGs in cervical cancer.

MATERIALS AND METHODS

Tissue Samples and DNA Extraction

Twenty-five fresh-frozen cervical cancer tissue samples were obtained from the Faculty of Medicine, Chulalongkorn University. All specimens were histologically proven malignant cells by pathologists. Samples were then divided into 2 subgroups. One set was fixed in formalin and then submitted for routine pathological examination. The second was preserved in tissue-freezing medium for use in further experiments, as shown in Figure 1. This arm of the experimental design was approved by the Human Research Ethics Committee of Chulalongkorn University. Clinical stage was retrieved from the clinical record data sheet. We classified International Federation of Gynecology and Obstetrics (FIGO) stages I-IIA as low-stage cervical cancer and FIGO stages IIB-IV as high-stage cervical cancer. For the histological grade, we reviewed slides and classified them into keratinized and nonkeratinized squamous cell carcinoma.

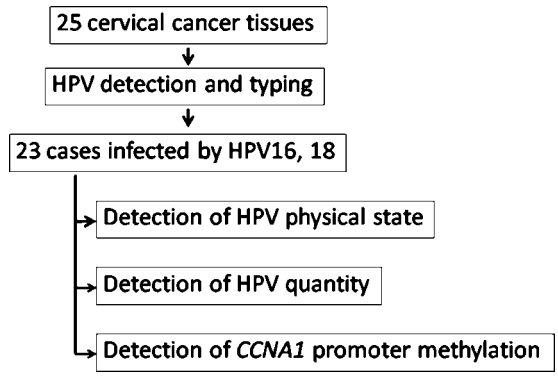

FIGURE 1. Flow chart of experiments.

DNA from cervical cancer samples was extracted by a standard technique with a lysis buffer (0.75-mol/L NaCl, 0.024-mol/L EDTA, pH 8.0) that was mixed with 10% sodium dodecyl sulfate and 20-mg/mL proteinase K and incubated at 50°C overnight, followed by phenol/chloroform and ethanol precipitation. After washing, the DNA samples were air-dried and resuspended in 50-μL distilled water.

HPV Detection and Typing

HPV *L1* and *E6* amplication and dot blot hybridization were performed as previously described.[18] Briefly, each L1 amplification reaction contained the L1 degenerate primers MY11 and MY09. The E6 reactions contained WD72, WD66, WD154, WD67, and WD76. Both reactions were used to amplify genomic DNA throughout 40 PCR cycles. Then the amplicons were further analyzed for the presence of high-risk HPV by dot blot hybridization using the HPV type-specific oligo probes WD170, WD132, RR1, RR2, WD103, WD165, WD, consensus L1, MY12/13, WD126, WD128, MY16, WD133/134, MY14, and WD174. The membranes were subjected to analysis by a phosphoimager. Results for L1 and E6 dot blots were scored independently. Duplicate filters were prepared for all specimens.

HPV Quantitation

For quantitation of HPV in cervical cancer samples, we used the *L1* as the representative gene of the HPV genome as well as *HAT* (histone acetyl transferase, which is the housekeeping gene) as the representative gene of human genome. All HPV DNA and tumor DNA in cervical cancer samples were measured semiquantitatively by performing duplex L1 and HAT primer PCR and repeating the above PCR reaction. The primer for *L1* was performed by MY09 and MY11. The primer for *HAT* was composed of HAT-F

(3′GGATGGTGAAAAATTGTCAT5′) and HAT-R (3′TTGG TAAACTTGAGGGATAT5′). The ratio between viral and host genome copy numbers was calculated from the relative intensity of PCR bands using ImageQuaNT software (Molecular Dynamics, Sunnyvale, Calif).

Detection of HPV's Physical State

To detect integration of HPV types 16 and 18, the *E2* region was amplified using primers as previously described.[18] Briefly, the *PIGR* gene was used as a control. The PCR reaction contained 1× PCR buffer (Qiagen, Tokyo, Japan), 0.2-mmol/L deoxynucleotide triphosphates, 0.5-μmol/L primers, 1 U of HotStar Taq (Qiagen), and 100-ng/μL DNA. The PCR condition was initially denatured for 15 minutes at 95°C, followed by 40 cycles at 95°C for 1 minute, at 45°C (for *E2*) and 55°C (for *PIGR)* for 1 minute, at 72°C for 1 minute, and with a final extension at 72°C for 7 minute. The product sizes of *E2* of HPV types 16 and 18 and *PIGR* were 1026 base pairs (bp), 1028 bp, and 1392 bp, respectively.

CCNA1 Promoter Methylation Detection

The DNA samples were subjected to bisulfite treatment. Briefly, 2 μg of genomic DNA was denatured in 0.2-mol/L sodium hydroxide. Subsequently, 10-mmol/L hydroquinone (Sigma-Aldrich, St Louis, Mo) and 3-mol/L sodium bisulfite (Sigma-Aldrich) were added and incubated at 50°C for 16 to 20 hours. The modified DNA was then purified using Wizard DNA purification resin (Promega, Madison, Wis), followed by ethanol precipitation.

Duplex methylation-specific primer polymerase chain reactions were performed to identify the *CCNA1* methylation level of all 23 samples infected by HPV 16 and 18, as previously described.[14] Polymerase chain reaction band intensity was measured using a phosphoimager. Methylated bands were observed at 46 bp and unmethylated bands at 64 bp. The *CCNA1* methylation percentage was calculated by a calibration experiment as previously described.[14] Some *CCNA1* methylation-positive bands were subsequently sequenced by a cloning and sequencing method.[14]

Statistical Analysis

Pearson correlation was used to determine the correlation between the quantity of HPV and *CCNA1* promoter methylation. Independent sample *t* test was performed for analyzing the effect of the physical state of HPV on *CCNA1* promoter methylation, the effect of HPV quantity, and the effect of *CCNA1* methylation on the stage and grade of cervical cancer.

RESULTS

HPV Detection and Typing

All 25 cervical cancer samples were infected by HPV. To observe HPV typing using L1- and E6-specific probes, 23 cases could be detected of HPV types 16 and 18, whereas 2 samples were infected by unclassifiable HPV types. Of the 23 classifiable tissue samples, 16 and 7 samples were infected by HPV 16 and 18, respectively.

HPV Quantitation

Twenty-three cervical cancer tissue samples containing HPV types 16 and 18 were evaluated for quantity of HPV by measuring the percentage of intensity of L1 per HAT bands. All 23 samples had a high quantity of HPV: the lowest was 58% and the highest was 94.5%.

HPV's Physical State

The positive band of the *E2* gene was investigated in the samples infected by the episomal form of HPV. This band was not found in samples infected by the integrated form. From the 23 samples infected by HPV types 16 and 18, 13 samples were found in integrated form, whereas 10 samples were found in episomal form.

CCNA1 Promoter Methylation

All 23 samples indicated *CCNA1* promoter methylation. Each sample had both methylated and unmethylated bands. The range of the intensity percentage of methylated bands was from 5% to 79%.

HPV Quantity and *CCNA1* Promoter Methylation

To investigate the correlation between the quantity of HPV and *CCNA1* promoter methylation, the proportion of L1/HAT and the percentage intensity of *CCNA1* promoter methylation were observed. No statistically significant correlation was found between the quantity of HPV and *CCNA1* promoter methylation.

HPV's Physical State and *CCNA1* Promoter Methylation

The effect of HPV form and *CCNA1* methylation level in cervical cancer tissues was observed. Cervical cancer tissues infected by the integrated form of HPV had a significantly higher percentage intensity of *CCNA1* methylation

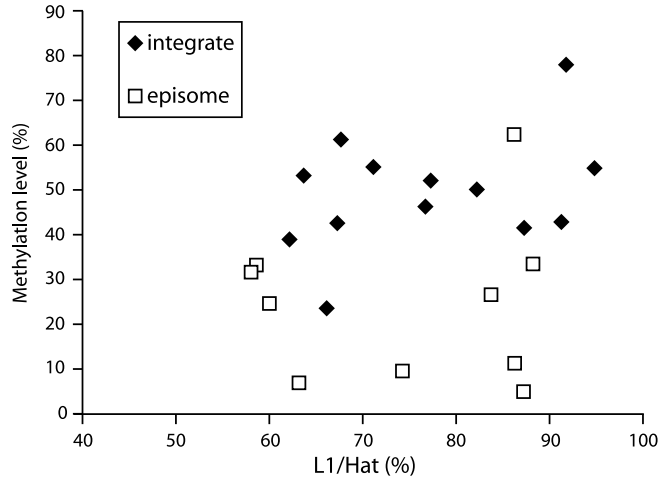

FIGURE 2. Graphical comparison between the proportions of L1/HAT (%; x-axis) and *CCNA1* promoter methylation level (%; y-axis).

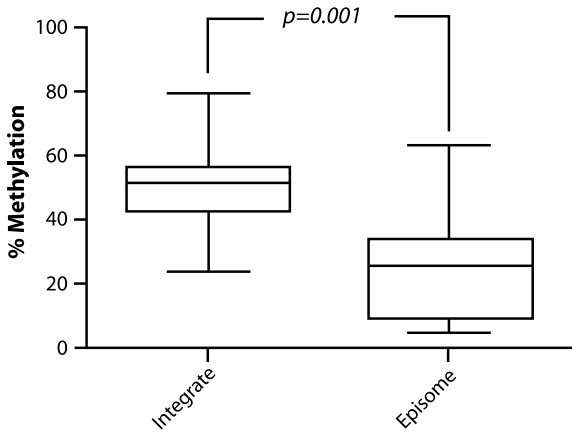

FIGURE 3. Graphical comparison between HPV physical state (x-axis) and *CCNA1* promoter methylation levels (y-axis).

than tissues that contained the episomal form of HPV (P = 0.001; 95% CI, 11.96–38.44), as shown in Figures 2 and 3.

HPV Quantity on Clinical Stage and Histological Grade

The effect of HPV quantity in cervical cancer tissues on the stage and grade of these samples was observed. There was no statistically signification correlation both between HPV quantity and stage (P = 0.137) and between the HPV quantity and the grade (P = 0.703) as shown in Figure 4.

CCNA1 Promoter Methylation on Clinical Stage and Histological Grade

The effect of *CCNA1* promoter methylation on the stage and grade of these samples was investigated. There was no statistically significant correlation both between the

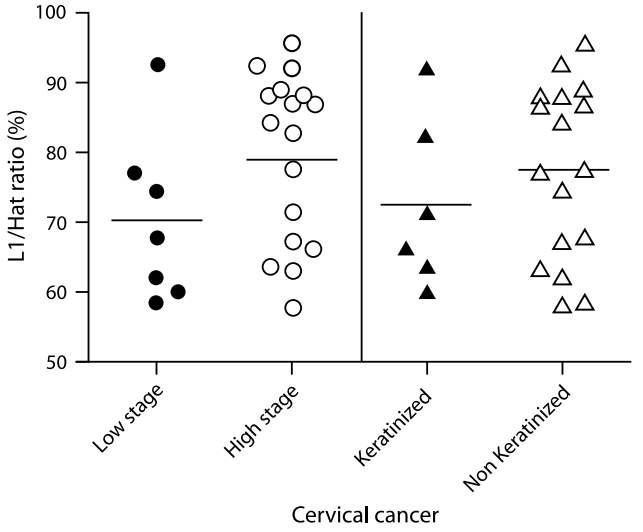

FIGURE 4. Graphical comparison between the proportions of L1/HAT (%; y-axis) and clinical stage and histological grade (y-axis).

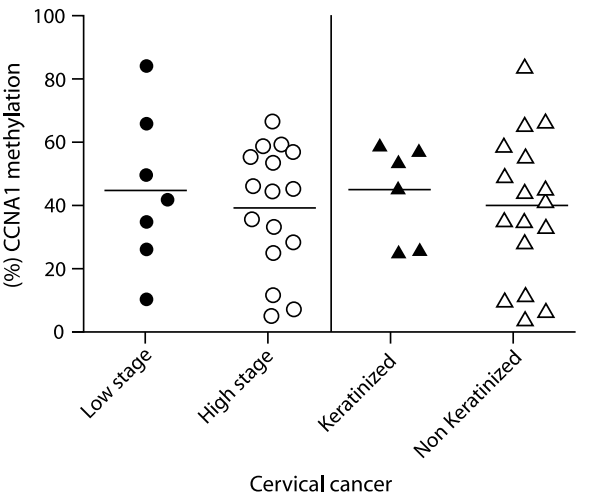

FIGURE 5. Graphical comparison between *CCNA1* promoter methylation levels (%; y-axis) and clinical stage and histological grade (y-axis).

CCNA1 promoter methylation and the stage (P = 0.610) and between the *CCNA1* methylation and the grade (P = 0.245) as shown in Figure 5.

DISCUSSION

Almost 100% of patients with cervical cancer have been infected by HPV, with the highest risk of this cancer found in HPV types 16 and 18.[2] Human papillomavirus can lead to tumorigenesis by several pathways, such as the mechanism of HPV E6, which binds and leads to degradation of the p53 tumor-suppressor protein, whereas HPV E7 competes in the interaction of retinoblastoma (Rb) with E2F, driving cell cycle progression.[4] In addition, TSG dysfunction also can lead to tumorigenesis. Loss of heterozygosity and promoter methylation are the main risk factors of TSG dysfunction. Our previous study showed a very high percentage of *CCNA1* promoter methylation in cervical cancer tissues, whereas no methylation was observed in normal tissues.[14] These results indicated that *CCNA1* functioned as a TSG in cervical cancer. Together with HPV signifying a high risk of this cancer, this encouraged us to determine the effect of the correlation between HPV and *CCNA1* promoter methylation.

In this study, we chose only cervical cancer tissues that were infected by very high-risk HPV: types 16 and 18. The data clearly expressed that HPV quantity had no effect on *CCNA1* promoter methylation; therefore, we can conclude that the quantity of HPV infection is not important for *CCNA1* methylation. In contrast, the physical state of HPV had a strong potential effect. We detected a significantly higher percentage intensity of *CCNA1* promoter methylation in cervical cancer tissues infected by the integrated rather than the episomal form of HPV. In the episomal form, E2 suppresses E6 and E7 transcription, whereas in the integrated form, E2 is disrupted, leading to up-regulation of E6 and E7.[4] Taken together, there may also be other roles of E6 and E7 in tumor development. Not only the function of p53 in the degradation role of E6 and interaction with the Rb role of

E7, but E7 might also have some ability to form a complex with DNA methyltransferase on the *CCNA1* promoter, leading to promoter methylation of this gene. This event might cause tumorigenesis because of lost function of cyclin A1 encoded by *CCNA1*.

Notably, although there are data proposing that integrated forms of HPV could activate the mechanism causing progression from preinvasive advance lesions to invasive carcinoma,[2] no correlation between the viral copy number and clinical stage were found,[19] although a dose-response association between viral load and precancerous lesion grade may be implicated.[8] In this study, we also found no significant correlation between HPV quantity and clinical staging. From our knowledge, we hypothesized that cells infected by only one integrated HPV could have the same outcome as those cells with multiple viral copies with regard to tumor progression but not tumor initiation. In addition, *CCNA1* promoter methylation can occur in those cells displaying HPV integration, albeit with one copy. This is likely due to E7 augmentation and interaction between E7 and DNA methyltransferase 1. This event can eventually transform precancerous to cancer, but this line of evidence warrants further investigation to essentially explore mechanisms of integrated HPV and *CCNA1* promoter methylation in promoting tumor progression.

In our previous study,[14] we observed *CCNA1* methylation and expression in HeLa and SiHa, which are cervical cancer cell line infecting with HPV. We found that methylation had controlled expression of this gene. In the present study, we found the samples with episomal form had lower percentage of *CCNA1* methylation than the samples with integrated form. This implied that there was higher *CCNA1* expression in samples with episomal form than in samples with integrated form. *Cyclin A1* plays a role as tumor suppressor gene in cervical cancer, head and neck cancer, and nasopharyngeal carcinoma.[14–16] Its function as TSG is involved in DNA repair.[20] Cyclin A1 may lose its function to repair DNA damage in cervical cancer infected by integrated form more than in episomal form.

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REVIEW

Long interspersed nuclear element-1 hypomethylation in cancer: biology and clinical applications

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Abstract Epigenetic changes in long interspersed nuclear element-1s (LINE-1s or L1s) occur early during the process of carcinogenesis. A lower methylation level (hypomethylation) of LINE-1 is common in most cancers, and the methylation level is further decreased in more advanced cancers. Consequently, several previous studies have suggested the use of LINE-1 hypomethylation levels in cancer screening, risk assessment, tumor staging, and prognostic prediction. Epigenomic changes are complex, and global hypomethylation influences LINE-1s in a generalized fashion. However, the methylation levels of some loci are dependent on their locations. The consequences of LINE-1 hypomethylation are genomic instability and alteration of gene expression. There are several mechanisms that promote both of these consequences in *cis*. Therefore, the methylation levels of different sets of LINE-1s may represent certain phenotypes. Furthermore, the methylation levels of specific sets of LINE-1s may indicate carcinogenesis-dependent hypomethylation. LINE-1 methylation pattern analysis can classify LINE-1s into one of three classes based on the number of methylated CpG dinucleotides. These classes include hypermethylation, partial methylation, and hypomethylation. The number of partial and hypermethylated loci, but not hypomethylated LINE-1s, is different among normal cell types. Consequently, the number of hypomethylated loci is a more promising marker than methylation level in the detection of cancer DNA. Further genome-wide studies to measure the methylation level of each LINE-1 locus may improve PCR-based methylation analysis to allow for a more specific and sensitive detection of cancer DNA or for an analysis of certain cancer phenotypes.

Keywords Long interspersed nuclear element-1s · DNA methylation · Hypomethylation · Partial methylation · Cancer · LINE-1

Because of the retrotransposition events that have occurred during evolution, the human genome contains more than 500,000 long interspersed nuclear element-1 (LINE-1 or L1) copies (Lander et al. 2001). Most LINE-1s are truncated. More than 10,000 LINE-1s are longer than 4.5 kb and consist of a 5′ untranslated region (UTR), two open reading frames, and a 3′ UTR containing a polyadenylation signal (Penzkofer et al. 2005). The DNA methylation levels of LINE-1 5′ UTRs in cancer have been extensively evaluated for potential use as an epigenomic marker for cancer (Chalitchagorn et al. 2004). The mean level of LINE-1 methylation in most cancer types is lower than in normal cells (Table 1). The degree of LINE-1 hypomethylation increases in more advanced cancers (Table 2 and Electronic supplementary material (ESM) Table 1). The methylation of other interspersed repetitive sequences (IRSs), such as Alu elements and human endogenous retrovirus (HERV) sequences, has been evaluated to a lesser extent (Tables 1 and 2 and ESM Table 1). LINE-1 and other IRS methylation levels have the potential to be used as universal tumor markers for

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Table 1 Interspersed repetitive sequence hypomethylation in cancer

Type of cancer	Repeated sequence	Hypomethylation	Reference
Abdominal paragangrioma	LINE-1	Yes	Geli et al. (2008)
Breast cancer	Alu	Yes	Cho et al. (2010)
	LINE-1	Yes	Cho et al. (2010)
Cervical cancer	LINE-1	Yes	Shuangshoti et al. (2007)
Cholangiocarcinoma	LINE-1	Yes	Kim et al. (2009a)
Colorectal cancer	Alu	Yes	Kwon et al. (2010; Rodriguez et al. (2008)
	LINE-1	Yes	Chalitchagorn et al. (2004; Suter et al. (2004); Matsuzaki et al. (2005); Estecio et al. (2007); Iacopetta et al. (2007); Ogino et al. (2008a); Nosho et al. (2009a, b); An et al. (2010); Baba et al. (2010); Ibrahim et al. (2011); Irahara et al. (2010); Kawakami et al. (2011); Kwon et al. (2010)
Ependymoma	Alu	Yes	Xie et al. (2010)
Esophagus cancer	LINE-1	Yes	Chalitchagorn et al. (2004)
Gastric cancer	Alu	Yes	Yoo et al. (2008); Park et al. (2009); Hou et al. (2010); Xiang et al. (2010); Yoshida et al. (2011)
	LINE-1	Yes	Chalitchagorn et al. (2004; Yoo et al. (2008); Park et al. (2009); Yoshida et al. (2011)
Germ cell tumor	LINE-1	Yes	Alves et al. (1996)
Fibrolamellar carcinoma of liver	LINE-1	No	Trankenschuh et al. (2010)
Head and neck squamous cell cancer	LINE-1	Yes	Chalitchagorn et al. (2004); Hsiung et al. (2007); Smith et al. (2007); Subbalekha et al. (2009)
Hepatoma	Alu	Yes	Lee et al. (2009)
	LINE-1	Yes	Takai et al. (2000); Chalitchagorn et al. (2004); Tangkijvanich et al. (2007); Lee et al. (2009); Kim et al. (2009b); Formeister et (al. 2010); Trankenschuh et al. (2010)
Leukemia (acute promyelocytic leukemia)	Alu Yb8	No	Choi et al. (2009)
	LINE-1	No	Choi et al. (2009)
Leukemia (chronic myelogenous leukemia)	Alu	Yes	Roman-Gomez et al. (2008); Fabris et al. (2011)
	LINE-1	Yes	Roman-Gomez et al. (2008); Roman-Gomez et al. (2005); Fabris et al. (2011)
Leukemia (plasma cell leukemia)	LINE-1	Yes	Bollati et al. (2009)
Lung cancer (non-small cell lung cancer)	Alu	Yes	Daskalos et al. (2009)
	LINE-1	Yes	Chalitchagorn et al. (2004); Daskalos et al. (2009); Jin et al. (2009); Saito et al. (2010)
Lymphoma	LINE-1	No	Chalitchagorn et al. (2004)
Malignant peripheral nerve sheath tumor	LINE-1	No	Feber et al. (2011)
Melanoma	LINE-1	Yes	Tellez et al. (2009)
Multiple myeloma	Alu	Yes	Bollati et al. (2009)
	LINE-1	Yes	Bollati et al. (2009)
Neuroendocrine tumor	Alu	Yes	Choi et al. (2007)
	LINE-1	Yes	Choi et al. (2007)
Neurofibromatosis	LINE-1	No	Feber et al. (2011)

Table 1 (continued)

Type of cancer	Repeated sequence	Hypomethylation	Reference
Ovarian cancer	AluHER	Yes	Watts et al. (2008)
	V-W	Yes	Menendez et al. (2004)
	LINE-1	Yes	Menendez et al. (2004); Pattamadilok et al. (2008); Woloszynska-Read et al. (2008); Dammann et al. (2010)
Parathyroid adenoma	LINE-1	No	Juhlin et al. (2010)
Pheochromocytoma	LINE-1	Yes	Geli et al. (2008)
Prostate cancer	Alu	Yes	Kim et al. (2011)
	LINE-1	Yes	Santourlidis et al. (1999; Schulz et al. (2002); Chalitchagorn et al. (2004); Florl et al. (2004); Kindich et al. (2006); Yegnasubramanian et al. (2008); Cho et al. (2009)
Renal cell carcinoma	LINE-1	No	Florl et al. (1999); Chalitchagorn et al. (2004)
Thyroid cancer (follicular type)	LINE-1	No	Lee et al. (2008)
Thyroid cancer (papillary type)	LINE-1	No	Chalitchagorn et al. (2004)
Urothelial cancer	HERV-K	Yes	Florl et al. (1999)
	Alu Yb8	Yes	Choi et al. (2009)
	LINE-1	Yes	Jurgens et al. (1996); Florl et al. (1999); Neuhausen et al. (2006); Choi et al. (2009); Wilhelm et al. (2010); Wolff et al. (2010)

the detection of cancer DNA and to predict prognosis (Watanabe and Maekawa 2010).

LINE-1s have often been referred to as parasitic or junk DNA sequences. However, many LINE-1s play a role in gene regulation, and this control is regulated by the 5′ UTR methylation level (Aporntewan et al. 2011). As a result, changes in the methylation status of different sets of LINE-1 loci may lead to different cellular phenotypes (Phokaew et al. 2008; Aporntewan et al. 2011). These differences may be an underlying reason why LINE-1 methylation levels in normal cells show so much variation (Chalitchagorn et al. 2004). Lower methylation levels can also be found in many non-malignant conditions. Current PCR-based techniques were designed to measure LINE-1 methylation level and cannot distinguish between malignant- and non-malignant-associated LINE-1 hypomethylation (Xiong and Laird 1997; Laird 2010; Weisenberger et al. 2005; Yang et al. 2004). Therefore, a technique that measures not only the level but also the pattern of LINE-1 methylation should improve detection specificity and sensitivity and broaden the applications of this tumor marker. The topics of this review therefore include the following: (1) an up-to-date review of studies on LINE-1 and other IRS methylation levels in cancer; (2) the characteristics of LINE-1 hypomethylation in cancer; (3) the locus-dependent roles of LINE-1 hypomethylation in cancer development; and (4) the improvement in cancer DNA identification by LINE-1 methylation classification.

LINE-1 and other IRS methylation levels in cancer

The methylation levels of LINE-1s, Alu elements, and some types of HERVs have been studied (Table 1). LINE-1 is the IRS element that is most frequently studied, and its hypomethylation has been found in many cancers. In a few cancer types, including cancer of the kidney, thyroid, and lymph nodes; acute promyelocytic leukemia; malignant peripheral nerve sheath tumor; and parathyroid adenoma, LINE-1 hypomethylation had not been found (Table 1). LINE-1 hypomethylation is also found in premalignant lesions of the cervix (Shuangshoti et al. 2007), extrahepatic bile duct (Kim et al. 2009a), and stomach (Park et al. 2009). Unexpectedly, LINE-1 hypermethylation was observed in some lesions that possess a high potential for malignant transformation, including lesions associated with myelodysplastic syndrome (Romermann et al. 2007) and liver cirrhosis (Takai et al. 2000). Interestingly, LINE-1 hypermethylation is found in partial hydatidiform moles, whereas

LINE-1 hypomethylation is seen in triploid diandric embryos. Both lesions originate from dispermic fertilization of an oocyte, suggesting that LINE-1 hypermethylation in moles is directly linked to the neoplastic process and is not a consequence of growth control (Perrin et al. 2007).

As shown in Table 2 and ESM Table 1, LINE-1 hypomethylation is associated with advanced tumor stage, higher histological grade, and poor prognosis. LINE-1 hypomethylation increases with tumor size (Tangkijvanich et al. 2007) and with higher tumor stage (Florl et al. 1999; Kindich et al. 2006; Pattamadilok et al. 2008; Lee et al. 2009; Baba et al. 2010). With increasing histological grade, according to multistep carcinogenesis, LINE-1 hypomethylation levels are increased in many cancer types (Florl et al. 1999; Shuangshoti et al. 2007; Cho et al. 2007; Park et al. 2009; Iramaneerat et al. 2011; Pattamadilok et al. 2008). Furthermore, LINE-1 hypomethylation is correlated with chromosomal aberrations (Schulz et al. 2002; Cho et al. 2007; Choi et al. 2007; Ogino et al. 2008a; Bollati et al. 2009), the hypermethylation of tumor suppressor genes (Choi et al. 2007; Kim et al. 2009a), mutations of tumor suppressor genes (Iacopetta et al. 2007; Kim et al. 2009a), the alternate transcription of oncogenes (Wolff et al. 2010), and the deregulation of cancer genes (Woloszynska-Read et al. 2008). Therefore, LINE-1 hypomethylation is associated with malignant phenotypes in human cells, deregulating gene expression and accelerating DNA rearrangement. Interestingly, the LINE-1 hypomethylation level is inversely associated with microsatellite instability (Estecio et al. 2007; Iacopetta et al. 2007; Ogino et al. 2008a; Goel et al. 2010; Kawakami et al. 2011). This finding may indicate that microsatellite instability and LINE-1 hypomethylation are characteristics of different genomic instability mechanisms.

From a clinical point of view, LINE-1 hypomethylation is associated with tumor metastasis (Schulz et al. 2002; Choi et al. 2007), the recurrence rate (Formeister et al. 2010), and the mortality rate (Ogino et al. 2008b; Ahn et al. 2011). LINE-1 hypomethylation has been reported to be a prognostic marker in several types of cancer including the stage IA subgroup of non-small cell lung cancer (Saito et al. 2010), ovary (Pattamadilok et al. 2008), and colon (Ogino et al. 2008b; Baba et al. 2010). LINE-1 hypomethylation has been proposed to be used as a screening tool for cancer detection. LINE-1 hypomethylation is observed in blood leukocyte DNA (Hsiung et al. 2007; Wilhelm et al. 2010), serum (Chalitchagorn et al. 2004; Tangkijvanich et al. 2007), and oral rinse samples (Subbalekha et al. 2009). Moreover, LINE-1 hypomethylation has also been demonstrated to be a surrogate marker for predicting tumor treatment response and prognosis (Aparicio et al. 2009; Sonpavde et al. 2009; Bernstein et al. 2010; Fang et al. 2010; Kawakami et al. 2011).

Alu elements and HERV genomes have been studied less frequently (Table 1). Hypomethylation of Alu sequences was reported in nine cancers, whereas hypomethylation of HERV-K and HERV-W genomes was found in urothelial cancer (Florl et al. 1999) and ovarian cancer (Menendez et al. 2004), respectively. All of the Alu- and HERV-hypomethylated cancers also possess LINE-1 hypomethylation. Certain cancer phenotypes are associated with the methylation levels of certain IRS types. For example, HERV-K, but not LINE-1 and HERV-E, methylation levels are associated with poor prognosis and platinum resistance of ovarian clear cell carcinoma (Iramaneerat et al. 2011).

Characteristics of LINE-1 and global hypomethylation in cancer

Transgenic mice with hereditary defects in DNA methyltransferase show increased risk of developing cancer (Gaudet et al. 2003). Therefore, global hypomethylation may be one of the mechanisms that promote carcinogenesis and is unlikely to be just a consequence of cancer development. However, lower genome-wide methylation levels have also been found in many conditions, such as embryogenesis (Migeon et al. 1991; Kremenskoy et al. 2003), aging (Lutz et al. 1972; Gonzalo 2010), congenital malformation (Wang et al. 2010), exposure to certain environments (Bollati et al. 2007), nutrition (Brunaud et al. 2003), and autoimmune diseases (Richardson et al. 1990). There is no report of increased cancer development risk in individuals with some of these conditions. Therefore, it is reasonable to hypothesize that the genomic distribution of IRS methylation levels is different in global hypomethylation-related conditions. Interestingly, in some conditions, the loss of genome-wide methylation is IRS type-specific. For example, hypomethylation of Alu elements and HERV-K, but not LINE-1, was found in aging cells (Jintaridth and Mutirangura 2011). However, LINE-1 hypomethylation has been demonstrated in many other conditions (Schulz et al. 2006). Because LINE-1 methylation levels can regulate host gene expression in *cis* (Aporntewan et al. 2011), it is reasonable to hypothesize that the reduction in LINE-1 methylation is the result of epigenomic heterogeneity. A simpler explanation is that even though two different cells possess the same number of LINE-1 loci and methylation levels, each LINE-1 locus may have a different level of LINE-1 methylation in these cells (Phokaew et al. 2008). Therefore, LINE-1 hypomethylation is a cancer biomarker that may be a diagnostic tool for many cancer types. However, LINE-1 hypomethylation is not specific to cancer. The inclusion of information regarding the genomic LINE-1 methylation distribution pattern should therefore be a promising way to improve and

Table 2 Interspersed repetitive sequence hypomethylation and cellular, molecular phenotype

Cancer	IRS	Cellular phenotype			Molecular association	Reference
		Higher clinical stage	Poorer histological grade	Survival		
Cervical cancer	L1	NR	PE	NR	NR	Shuangshoti et al. (2007)
Cholangiocarcinoma	L1	NR	PE	NR	PE for CIMP and TSG mutation	Kim et al. (2009a)
Colorectal cancer	L1	NR	PE	NR	NR	Chalitchagorn et al. (2004)
	L1	NR	NR	NR	PE for MSS	Matsuzaki et al. (2005)
	L1	NR	NR	NR	PE for MSI and CIN	Deng et al. (2006)
	L1	NR	NR	NR	NE for MSI	Estecio et al. (2007)
	L1	PE	PE in mucinous histology	NR	NE for MSI and TSG mutation	Iacopetta et al. (2007)
	L1	NR	NR	NR	NE for MSI and CIMP PE for chromosomal alteration in non-MSI tumor	Ogino et al. (2008a)
	L1	NR	NR	PE	NR	Ogino et al. (2008b)
	L1	NR	NR	NR	NE for SNPSs in one-carbon pathway genes.	Hazra et al. (2010)
	L1	NR	NR	NR	LINE-1 methylation level correlated between synchronous cancer pairs from the same individuals.	Nosho et al. (2009a)
	L1	NR	NR	NR	NE for *DNMT3B*- positive tumors	Nosho et al. (2009b)
	L1	NR	NR	NR	PE for CIMP	An et al. (2010)
	L1	NR	NR	PE in proximal colon cancer NE in distal colon cancer	NR	Ahn et al. (2010)
	L1	NR	NR	PE	PE for MSI, CIMP, CIN, TSG mutation and TSG expression	Baba et al. (2010)
	L1	NR	NR	NR	NE for MSI and methylation index PE for MSS HNPCC	Goel et al. (2010)
	L1	NR	PE	NR	NR	Ibrahim et al. (2011)
	L1	NR	NR	PE	NE for MSI and CIMP	Kawakami et al. (2011)
	Alu, L1	NE	PE	NR	NR	Kwon et al. (2010)
Ependymoma	Alu	NR	PE	NR	NR	Xie et al. (2010)
Gastric cancer	Alu	NR	PE	NR	NR	Park et al. (2009)
	L1	NR	PE	NR	NR	Park et al. (2009)
	L1	NR	NR	NR	PE for folate metabolizing gene polymorphisms	Hou et al. (2010)
Gastrointestinal stromal cancer	L1	PE	NR	NR	NR	Igarashi et al. (2010)
Head and neck cancer	L1	PE	NR	NR	NR	Smith et al. (2007)
	L1	NR	NR	PE especially HPV 16 negative SCC	NR	Furniss et al. (2008)
	L1	NS	NS	NR	NR	Subbalekha et al. (2009)

Table 2 (continued)

Cancer	IRS	Cellular phenotype			Molecular association	Reference
		Higher clinical stage	Poorer histological grade	Survival		
Hepatocellular carcinoma	L1	PE	PE	NR	NR	Tangkijvanich et al. (2007)
	Alu	NE	PE	NR	NR	Lee et al. (2009)
	L1	PE	PE	NR	NR	Lee et al. (2009)
	L1	NR	PE	NR	NR	Kim et al. (2009b)
	L1	NR	NR	PE	NR	Formeister et al. (2010)
Multiple myeloma (MM)	Alu	NR	PE	NR	NE for hyperdiploid MM	Bollati et al. (2009)
	L1	NR	PE	NR	PE for chromosomal translocation	Bollati et al. (2009)
Nerve tumor	L1	NR	NE	NR	NR	Feber et al. (2011)
Neuroendocrine tumor	Alu	NR	NE	PE	PE for TSG methylation	Choi et al. (2007)
	L1	NR	NE	PE	PE for chromosomal alteration and gene methylation	Choi et al. (2007)
Non-small cell lung cancer (NSCLC)	L1	NR	SCC > adenocarcinoma ($P<0.001$)	NR	NR	Jin et al. (2009)
	L1	NR	NR	PE	NR	Saito et al. (2010)
Odontogenic tumor	L1	NR	Ameloblastoma > KCOT ($P=0.001$)	NR	NR	Kitkumthorn and Mutirangura (2010)
Ovarian cancer	L1	NS	NE	PE	NR	(Pattamadilok et al. 2008)
	L1	NR	NR	NR	PE with TSG expression	Woloszynska-Read et al. (2008)
	L1	NR	NR	NR	PE for follow-up patients treated with decitabine ($P<0.001$)	Fang et al. (2010)
	L1	PE	NR	NR	PE for TSG methylation	Woloszynska-Read et al. (2011)
Ovarian clear cell carcinoma	L1	PE	NR	NR	NR	Iramaneerat et al. (2011)
	HERV-E	PE	NR	NR	NR	Iramaneerat et al. (2011)
	HERV-K	PE	NR	PE	NR	Iramaneerat et al. (2011)
Pancreatic cancer	L1	NR	NR	NR	PE for *MTHFR* polymorphisms	Matsubayashi et al. (2005)
Prostate cancer	L1	PE	NR	NR	NR	Santourlidis et al. (1999)
	L1	PE	NR	PE	PE with chromosomal aberration	Schulz et al. (2002)
	L1	PE	NR	NR	NR	Kindich et al. (2006)
	Alu	PE	PE	NR	NR	Cho et al. (2007)
	L1	PE	PE	NR	NR	Cho et al. (2007)
	L1	NR	NR	PE	NR	Yegnasubramanian et al. (2008)
	L1	NR	PE	NR	NR	Cho et al. (2009)

Table 2 (continued)

Cancer	IRS	Cellular phenotype			Molecular association	Reference
		Higher clinical stage	Poorer histological grade	Survival		
Urothelial cancer	L1	PE	PE	NR	NR	Florl et al. (1999)
	L1	PE	PE	NE	NR	Neuhausen et al. (2006)
	L1	NR	NR	NR	PE for *Met* oncogene alternate transcript	Wolff et al. (2010)

IRS interspersed repetitive sequence, *L1* long interspersed nucleotide element-1, *NR* no report, *NS* non-significant, *PE* positive evidence, *NE* negative evidence, *TSG* tumor suppressor gene, *CIMP* CpG island methylator phenotype, *MSS* microsatellite stable, *MSI* microsatellite instability, *CIN* chromosomal instability, *SNP* single nucleotide polymorphism, *DNMT3B* DNA methyltransferase-3B, *HNPCC* hereditary nonpolyposis colorectal cancer, *MM* multiple myeloma, *HCC* hepatocellular carcinoma, *SCC* squamous cell carcinoma, *KCOT* keratocystic odontogenic tumor, *HERV-E* human endogenous retrovirus E, *HERV-K* human endogenous retrovirus K, *MTHFR* methylenetetrahydrofolate reductase

widen the applications of LINE-1 methylation as a tumor marker (Pobsook et al. 2011).

Although LINE-1 methylation levels are variable in both cancer and normal cells, the mechanisms that alter methylation levels may be different. Normal cells possess several patterns of LINE-1 methylation levels. The levels of some cell types are precise and limited to within a specific range. In other cases, such as in the esophagus and thyroid, the ranges are expanded (Chalitchagorn et al. 2004). Similar patterns can be observed when the methylation status of each LINE-1 locus is observed (Phokaew et al. 2008). Different loci possess different methylation levels. Some are limited in range and others have wider ranges. Levels of LINE-1 locus methylation between different cell types are usually different, but each locus reveals similar patterns regarding the range of methylation levels (Phokaew et al. 2008).

Comparison of methylation levels between LINE-1 loci in normal cells showed no significant correlation. This result suggests that the methylation level is locus-dependent (Fig. 1; Phokaew et al. 2008). In contrast, significant associations of methylation levels between LINE-1 loci were frequently found in cancer. Therefore, the mechanism causing LINE-1 hypomethylation in cancer occurs generally and in a genome-wide manner (Fig. 1; Phokaew et al. 2008). However, this mechanism may be biased toward some IRS sequences. Using microarray analysis, Szpakowski et al. (2009) reported that primate-specific LINE-1 elements and most of the younger, primate-specific retroelements were preferentially hypomethylated in samples of squamous cell carcinoma of the head and neck in comparison to non-tumor adjacent tissue and normal controls. The association of the methylation level between two LINE-1 loci was found to be highest if they were located in the same gene (Phokaew et al. 2008). Therefore, in addition to evolutionarily derived classifications, LINE-1 hypomethylation in cancer can be influenced by genomic location.

LINE-1 methylation regulates gene expression in *cis*

The notion that LINE-1 is methylated to prevent the process of retrotransposition should be reevaluated. First, in the human genome, less than 100 LINE-1s are retrotransposition competent, and only a few LINE-1s have been shown to be responsible for retrotransposition events during human evolution (Sassaman et al. 1997). Although a recent study showed that LINE-1 retrotransposition may be common (Lupski 2010; Beck et al. 2010), this evidence fails to explain the methylation of the vast majority of retrotransposition-incompetent LINE-1s. The human genome possesses thousands of 5′ UTR-containing LINE-1s, and most of them are methylated to a certain degree (Chalitchagorn et al. 2004). It is unlikely that this

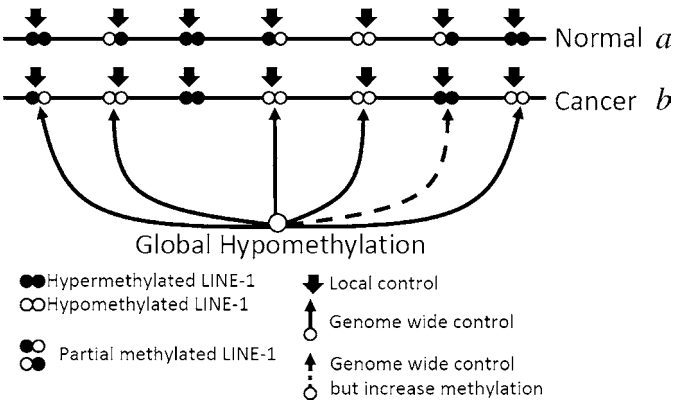

Fig. 1 Effect of global hypomethylation in cancer. *a* Normal genomes contain hypermethylated, partially methylated, and hypomethylated LINE-1s. The methylation levels of each locus are regulated in a location-dependent manner. *b* The cancer genome contains more hypomethylated LINE-1s. Global hypomethylation decreases the methylation status of many LINE-1 loci. However, there are some loci that are not influenced and some loci that show increased methylation levels. Local mechanisms are also present in cancer cells, and some locations are affected by the process of carcinogenesis

methylation provides a selective advantage to the cells by preventing retrotransposition. The significant differences in LINE-1 methylation levels between loci or cell types suggests that LINE-1 methylation may be important to maintain normal cellular function and that this function may be altered by the global hypomethylation process that occurs in cancer.

The location-dependent LINE-1 methylation pattern in normal cells suggests a role for epigenetic regulation. Currently, there are at least two reported mechanisms for how LINE-1 methylation regulates gene expression in *cis*. Both mechanisms are dependent on the transcriptional activity of the LINE-1 promoter. Moreover, similar to other promoters, the LINE-1 5′ UTR promoter is controlled by DNA methylation, and the transcription activity of a LINE-1 element is directly correlated with its hypomethylation level (Aporntewan et al. 2011). The first mechanism is that LINE-1-mediated control of gene expression is through the production of unique RNA sequences (Fig. 2). The other mechanism is that intragenic LINE-1 RNAs repress host gene expression via the nuclear RNA-induced silencing complex (RISC; Fig. 3).

There are two ways for the LINE-1 promoter to produce unique RNA sequences (Fig. 2). The 5′ UTR of LINE-1 is a promoter that transcribes in both the forward and reverse directions (Matlik et al. 2006; Weber et al. 2010; Speek 2001; Wolff et al. 2010; Rangwala et al. 2009). If the transcription is in the forward orientation, then the promoter produces LINE-1 RNA. However, the poly-A addition signal of LINE-1 does not always function. Consequently, many LINE-1 transcripts can continue beyond the end of the LINE-1 sequence, therefore resulting in 3′ transduction (Moran et al. 1999; Rangwala et al. 2009). These transduction sequences are unique RNA sequences generated by the LINE-1 promoter. On the other hand, LINE-1 5′ transduction that occurs by reverse transcription will also produce unique RNA sequences. A large number of these transduction sequences have been reported (Rangwala et al. 2009); however, there are currently only two examples that prove that these sequences are increased by LINE-1 hypomethylation (Weber et al. 2010; Wolff et al. 2010; Aporntewan et al. 2011).

Intragenic LINE-1 regulation of host gene expression was revealed by the finding that in vitro insertion of a full-length LINE-1 disrupted host gene expression (Han et al. 2004). In vivo, this gene regulation is tuned by LINE-1 methylation levels (Aporntewan et al. 2011). When LINE-1 methylation levels were reduced by chemical treatment or by carcinogenesis, a significant number of genes containing LINE-1s were repressed (Fig. 3a–c). The degree of this repression was inversely correlated with the intragenic LINE-1 methylation level. The role of LINE-1 methylation is to prevent the formation of a pre-mRNA–LINE-1–RNA complex. If the complex is formed, then the RISC protein AGO2 will bind and prevent mRNA production (Fig. 3; Aporntewan et al. 2011).

Comparative sequence analysis between intragenic and intergenic LINE-1s showed multiple conserved nucleotides in intragenic LINE-1s that are crucial for maintaining LINE-1 transcription and methylation (Aporntewan et al. 2011). Moreover, many LINE-1s are excluded from genomic regions containing housekeeping genes (Eller et al. 2007; Graham and Boissinot 2006). Therefore, locations of LINE-1s yield a selective advantage for human evolution. It is important to note that the diploid human genome contains an extensive amount of structural variation due to retrotransposition events (Huang et al. 2010; Ewing and Kazazian 2011). Consequently, variation in the expression of many genes may be due to the distinctive locations of heritable LINE-1s, and similar to other DNA polymorphisms, some LINE-1 insertions are polymorphisms that lead to certain disease-related phenotypes. LINE-1 hypomethylation may also control gene expression in *trans*. In some cancer cells, inhibition of LINE-1 reverse transcriptase can alter the expression of many genes (Carlini et al. 2010).

LINE-1 hypomethylation and genomic instability in cancer

In addition to a number of association studies (Ji et al. 1997; Lu and Randerath 1984; Daskalos et al. 2009), the high risk of chromosomal abnormalities in individuals with hereditary mutations in DNA methyltransferase genes indicates that global hypomethylation promotes genomic instability (Hansen et al. 1999; Eden et al. 2003). However, the underlying mechanisms of how DNA methylation

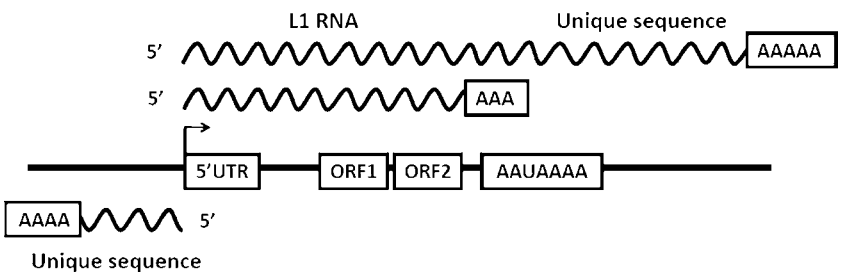

Fig. 2 LINE-1 can produce two types of unique RNA sequences. One type of unique sequence is the result of LINE-1 RNA transcription proceeding beyond the LINE-1 sequence. The other type occurs when the reverse LINE-1 promoter transcribes unique DNA sequences located beyond the 5′ end of LINE-1

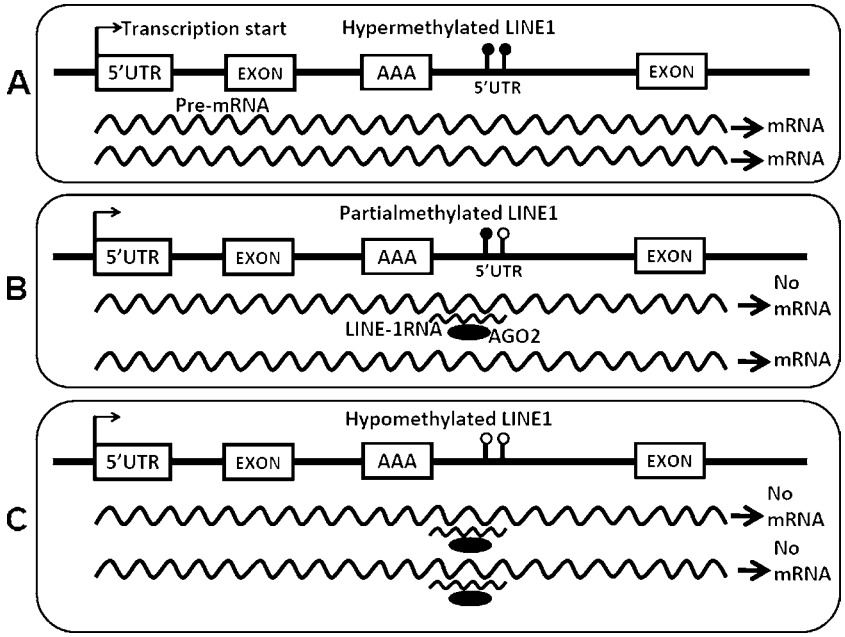

Fig. 3 Intragenic hypomethylated LINE-1s repress host gene expression via AGO2. The schematic demonstrates that the same gene from three different cells has different levels of intragenic LINE-1 methylation. **a** Hypermethylated LINE-1. **b** Partially methylated LINE-1. **c** Hypomethylated LINE-1. LINE-1 RNA is produced when the methylation of the LINE-1 5′ UTR is reduced. The LINE-1 RNA–pre-mRNA complex is bound by AGO2, and mRNA production is prevented

maintains genomic integrity are not yet known. Current reports suggest that LINE-1 hypomethylation leads to several events that promote genomic instability, including retrotransposition, endogenous DNA double-strand break (EDSB) repair, and the dysregulation of DNA repair genes.

The process of LINE-1 retrotransposition includes RNA transcription, protein translation, DNA restriction, reverse transcription, and integration (Moran 1999). This retrotransposition usually produces large DNA rearrangements (Huang et al. 2010; Gilbert et al. 2002). Recently, an advanced LINE-1 junction sequencing technique showed that somatic L1 insertions occur at high frequency in human lung cancer genomes (Iskow et al. 2010). Therefore, LINE-1 hypomethylation in cancer may increase the retrotransposition activity of some LINE-1s and consequently cause a faster rate of DNA rearrangement. However, many DNA rearrangements occur in cancer cells that are not LINE-1 retrotransposition events. Therefore, LINE-1 retrotransposition contributes to only a small proportion of mutations in cancer. Moreover, there are only a few reports that retrotransposition events can produce clonal expansion mutations (Miki et al. 1992). Finally, the loss of the methylation of non-retrotransposable repeats, such as satellite DNA, also promotes chromosome translocation (Maraschio et al. 1988; Ji et al. 1997). Therefore, LINE-1 retrotransposition may not be the major mechanism causing somatic mutation in cancer by global hypomethylation.

The second mechanism is the differential repair of methylated and unmethylated replication-independent EDSBs (RIND-EDSBs; Kongruttanachok et al. 2010). RIND-EDSBs are different from replication-dependent EDSBs and environmental- or radiation-induced DSBs. Replication-dependent EDSBs and radiation-induced DSBs, if unrepaired, lead to cell death. In contrast, RIND-EDSBs are ubiquitously present in all cells and always involve hypermethylation (Pornthanakasem et al. 2008). This occurrence indicates a time lag between methylated RIND-EDSB production and repair (Kongruttanachok et al. 2010). RIND-EDSBs can be produced within both methylated and unmethylated genomes. Methylated RIND-EDSBs are selectively repaired by the more precise ataxia telangiectasia mutated (ATM)-dependent non-homologous end joining repair process (Kongruttanachok et al. 2010). Therefore, the RIND-EDSB repair process of hypomethylated genomes is faster and more error-prone. Because the LINE-1 methylation levels of each locus are distinct, the mutation rate caused by RIND-EDSB repair errors is dependent on the methylation status of the genome near the EDSBs. Currently, there are only two reports focused on RIND-EDSBs (Pornthanakasem et al. 2008; Kongruttanachok et al. 2010). Further studies are needed to explore the causes and roles of RIND-EDSBs and to determine how genomic hypomethylation promotes instability.

A third possible mechanism is that LINE-1 hypomethylation down-regulates DNA repair genes. One of these genes is *PPP2R2B*, which contains intragenic LINE-1s. In cancer, these LINE-1s are frequently hypomethylated and *PPP2R2B* is frequently down-regulated (Aporntewan et al. 2011). One of the functions of PPP2R2B is to increase nuclear ATM protein (Suyarnsestakorn et al. 2010). ATM is a serine/threonine protein kinase that is important in the activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair, or apoptosis (Mavrou et al. 2008). A lack of ATM promotes genomic instability (Kim et al. 2002). Therefore, LINE-1 hypomethylation may indirectly promote genomic instability by interfering with ATM function.

LINE-1 methylation patterns in normal and cancer cells

It is commonly assumed that LINE-1 elements in normal cells are completely methylated. Combined bisulfite restriction analysis or COBRA, deep sequencing, and microarray analysis demonstrated that the genomic distribution of the methylation of LINE-1s and other IRS loci is not homogenous (Phokaew et al. 2008; Xie et al. 2009, 2011; Szpakowski et al. 2009). The methylation levels of LINE-1 loci can be divided into three groups: hypermethylated, partially methylated, and hypomethylated (Pobsook et al. 2011). Classification is based on the number of methylated and unmethylated CpG dinucleotides (Fig. 1). In normal cells, the majorities of LINE-1 loci are hypermethylated or partially methylated. Few LINE-1 loci are hypomethylated. Comparisons between normal white blood cells and normal oral epithelium showed that even though LINE-1 methylation levels are different, the number of hypomethylated loci was not distinguishable between the two normal tissues (Fig. 4). Therefore, the differences in methylation levels between normal cell types are primarily influenced by the number of hypermethylated and partially methylated loci. In cancer cells, the methylation of a majority of LINE-1 loci is decreased, with some loci remaining unchanged and a few being increased when compared with normal cells. Thus, the number of hypomethylated loci is increased in cancer cells (Fig. 4).

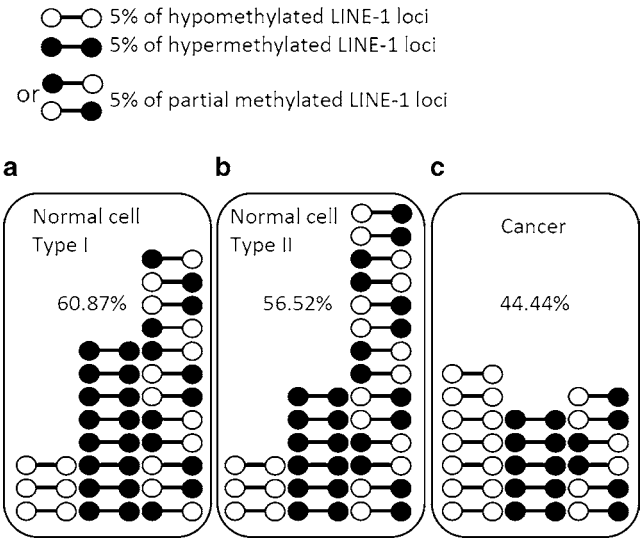

Fig. 4 Examples of LINE-1 methylation patterns in three cells. The number of LINE-1 loci and the methylation levels were approximated from the average levels of a previous report (Pobsook et al. 2011). Type I normal cells (**a**), type II normal cells (**b**), and cancer cells (**c**) possess LINE-1 methylation levels of 60.87%, 56.52%, and 44.44%, respectively. Even though different normal cell types contain different methylation levels, the numbers of partially methylated, hypermethylated, and hypomethylated loci were not different. Cancer cells showed lower methylation levels and a lower number of partially methylated loci, but a higher number of hypomethylated LINE-1 loci

A recent report showed distinctive characteristics of LINE-1 partial methylation that was dependent on malignant transformation (Pobsook et al. 2011). In normal cells, the number of partially methylated LINE-1 loci in each sample was directly correlated with the number of hypomethylated loci, but was inversely associated with the number of hypermethylated loci. This result suggested that a dynamic form of LINE-1 epigenetic modification, between partial methylation and hypermethylation, is present in normal cells. Because hypomethylated LINE-1s were not distinguishable between different types of normal cells, the dynamic between the partially methylated and hypermethylated forms may be the cause of the variation in LINE-1 methylation levels between normal cell types. Moreover, the more partially methylated loci may represent the lower LINE-1 methylation level. In contrast, in the cancer genome, the number of partially methylated LINE-1s was directly correlated with the number of hypermethylated LINE-1s. Therefore, in striking contrast to the normal genome, partially methylated LINE-1 loci represent a subset of methylated LINE-1s in cancer cells (Pobsook et al. 2011). Current PCR-based techniques, by real-time quantitative PCR, COBRA, and pyrosequencing, determine LINE-1 hypomethylation levels by combining all unmethylated CpG nucleotides from both partially methylated or hypomethylated loci (Xiong and Laird 1997; Laird 2010; Weisenberger et al. 2005; Yang et al. 2004). Therefore, the sensitivity in distinguishing cancer DNA is low. Pobsook et al. (2011) also showed that excluding partial methylation loci from the count of hypomethylated LINE-1 loci improved the sensitivity and specificity of cancer DNA detection.

From biology to clinical application and future direction of LINE-1 hypomethylation in cancer

Understanding how LINE-1 methylation levels change during multistep carcinogenesis has implications for diagnostic applications. Several LINE-1 and other IRS methylation studies have shown that global hypomethylation is a common epigenetic change in cancer (Table 1). Moreover, this process is directly correlated with cancer progression. Therefore, lower LINE-1 methylation levels have been shown to be associated with higher cancer stages and may also be a promising marker for the prognostic prediction of many cancers (Table 2 and ESM Table 1). Global methylation changes initiate early, and the genome becomes progressively hypomethylated during the process of multistep carcinogenesis. Therefore, LINE-1 and other IRS hypomethylation levels are candidate tumor markers for cancer (Table 2 and ESM Table 1).

There is a technical advantage to using PCR-based assays to measure IRS methylation levels. Multiple copies of IRSs are present in the genome; therefore, this detection method is highly sensitive even in poor-quality clinical DNA samples. These clinical samples include paraffin-embedded sections, plasma, and other fluid or washes, such as oral rinses (Chalitchagorn et al. 2004; Tangkijvanich et al. 2007; Aparicio et al. 2009; Subbalekha et al. 2009) (Vaissiere et al. 2009). LINE-1 hypomethylation was also detected in the white blood cells of cancer patients (Hsiung et al. 2007; Wilhelm et al. 2010). The source of the hypomethylated cells in cancer patients still needs to be identified to determine whether these cells are from cancer cells or from normal cells with systemic hypomethylated LINE-1s. Nevertheless, this evidence suggests that LINE-1 methylation is a promising marker in cancer risk prediction.

Cells must have a correct amount of LINE-1 methylation to maintain their physiological functions (Aporntewan et al. 2011). Consequently, there is a wide range of LINE-1 methylation levels found in normal cells, depending on cell type (Chalitchagorn et al. 2004). This methylation range leads to low specificity when using LINE-1 hypomethylation as a cancer screening marker. The ability to distinguish between normal and tumor DNA is low, particularly because clinical samples, including plasma, mouth washes, or Papanicolaou smears, are routinely contaminated with DNA from several normal cell types. LINE-1 methylation pattern analysis demonstrated unprecedented characteristics of LINE-1 partial methylation in normal cells and in the cancer global hypomethylation process (Pobsook et al. 2011). The interchangeable pattern between LINE-1 hypermethylation and partial methylation is a mechanism that may result in different LINE-1 methylation levels in normal cells (Pobsook et al. 2011). In cancer, global hypomethylation is observed because of the loss of methylation of previously hypermethylated and partial methylated loci. Most PCR-based LINE-1 methylation measurement techniques cannot differentiate unmethylated CpG dinucleotides of partially methylated LINE-1s from unmethylated LINE-1s. There was a recent report using COBRA to classify LINE-1s into the three classes. This report showed that the number of unmethylated LINE-1 loci was a more sensitive and specific marker than LINE-1 methylation level to detect cancer DNA in mouthwash samples (Pobsook et al. 2011). It may be interesting to compare the number of unmethylated LINE-1 loci with LINE-1 methylation levels in other clinical samples. Moreover, it may be worth exploring whether changes in partially methylated LINE-1 loci can be observed in, and are able to predict, malignant transformation in pathological lesions in the very early stages of carcinogenesis or tissues in patients at risk of developing cancer.

Although the methylation of a majority of LINE-1 loci is reduced in cancer, some loci are unchanged. Currently, there are several advanced genomic techniques, including deep sequencing (Xie et al. 2009; Xie et al. 2011) and custom-made microarrays (Szpakowski et al. 2009), that are capable of measuring the methylation level of each LINE-1 or IRS locus. These approaches identified certain classes of LINE-1s and IRSs that more frequently show loss of methylation in cancer. Improved deep sequencing techniques will be able to determine the proportions of the three LINE-1 methylation classes at each LINE-1 locus. It is important to reevaluate the clinical significance of LINE-1 methylation by these advanced techniques. These methods should help define the relevant LINE-1 locations, sequences, and methylation patterns that are specific to carcinogenesis. Moreover, some intragenic LINE-1 loci are methylated *cis*-regulatory elements of their host genes (Aporntewan et al. 2011). Altered expression of these genes may lead to certain cellular phenotypes and clinical presentations. Genome-wide arrays or deep sequencing may be used to design promising new sets of methylated LINE-1 PCR-based techniques specifically aimed for the classification of the epigenome of the tumor phenotype.

Interestingly, some pathological lesions with increased potential for malignant transformation, such as myelodysplastic syndrome lesions, liver cirrhosis, and partial hydatidiform moles, possess LINE-1 hypermethylation (Takai et al. 2000; Romermann et al. 2007; Perrin et al. 2007). Further descriptive studies of other lesions, genomic distributions, and methylation patterns will clarify in detail whether this epigenetic process occurs during the early steps of LINE-1 hypomethylation in cancer. It is important to note that genome-wide hypomethylation in cancer can result in hypermethylated LINE-1s at some loci (Fig. 4; Phokaew et al. 2008). If LINE-1 hypermethylation and hypomethylation are present at the same loci in premalignant tissues and cancer, this finding would be a breakthrough by showing that epigenomic changes precede genetic changes during carcinogenesis. Detailed molecular biological approaches to explain how LINE-1 methylation fluctuates from hypermethylation to hypomethylation will be important to understand the development of global hypomethylation in cancer.

Finally, global hypomethylation mechanisms may be crucial for future cancer prevention and treatment. Genome-wide hypomethylation is common, occurs at an earlier stage of carcinogenesis, and is still an active process in most cancers (Tables 1 and 2 and ESM Table 1). Global hypomethylation is an epigenomic process that leads to cellular phenotypic changes. LINE-1 hypomethylation in cancer alters the expression of a large number of genes. Therefore, this epigenomic alteration should be an important target for future cancer prevention strategies. More-

over, unlike mutation, hypomethylation is reversible. Therefore, global hypomethylation in cancer is a candidate for new cancer treatments in the future.

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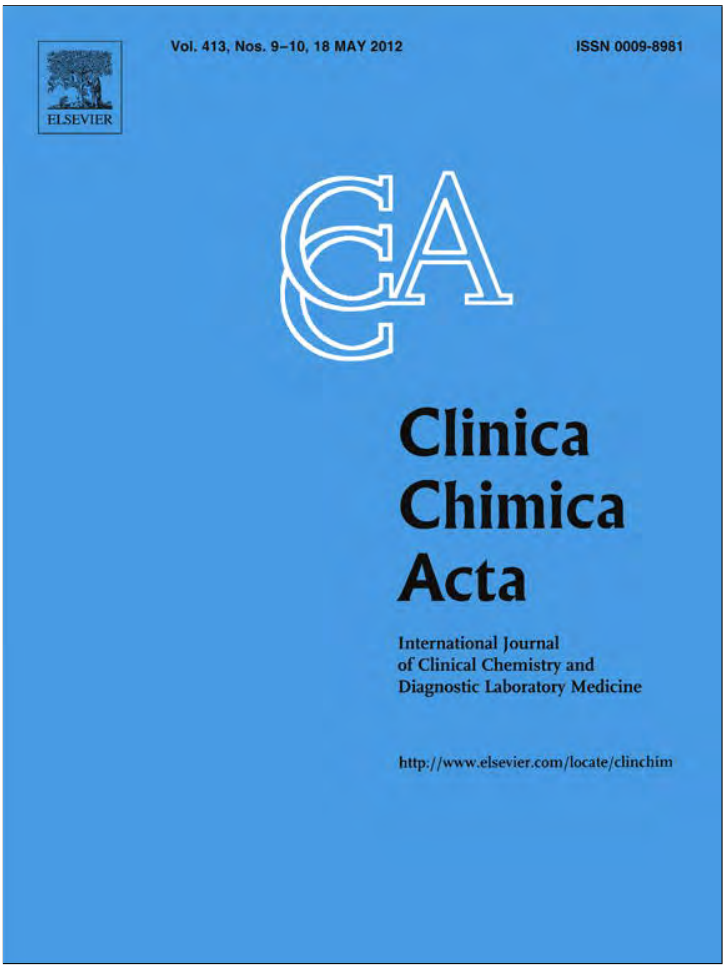

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LINE-1 methylation in the peripheral blood mononuclear cells of cancer patients ☆

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ABSTRACT

Background: Recently, we classified LINE-1 loci according to their methylation statuses and found that the percentage of hypomethylated LINE-1 loci ($^{u}C^{u}C$) can differentiate between the peripheral blood mononuclear cells (PBMCs) of oral cancer patients and normal controls with a higher specificity and sensitivity than overall methylation levels. Here, we evaluated the LINE-1 methylation levels and patterns in PBMCs from patients with cancers of the nasopharynx, lung, liver, bile duct, breast and colon.
Methods: Combined Bisulfite Restriction Analysis (COBRA) of LINE-1 loci was performed to examine the LINE-1 methylation statuses of PBMCs from 216 cancer patients with 6 different types of cancer compared with 144 normal controls.
Results: Only colorectal and nasopharyngeal cancer samples were found to have lower levels of overall LINE-1 methylation compared with normal controls ($p<0.0001$ and $p=0.0022$). However, $\%^{u}C^{u}C$ in cancers of the colon, liver, lung and nasopharynx was significantly higher compared with normal controls ($p<0.0001$, $p<0.0001$, $p=0.01$ and $p=0.001$, respectively). Furthermore, ROC curve analyses of these four cancer types also demonstrated the potential of $\%^{u}C^{u}C$ as a biomarker for cancer diagnosis.
Conclusion: Changes in the levels and patterns of genome-wide methylation of PBMCs are associated with cancer risk. For LINE-1, $\%^{u}C^{u}C$ is a more effective tumour marker for determining cancer risk than overall methylation levels.

1. Introduction

Epigenetic alterations are an important characteristic of carcinogenesis [1,2]. Hypermethylation of the promoters of specific genes and genome-wide DNA hypomethylation have been studied in most cancer types [3]. Genome-wide DNA hypomethylation is produced by the reduction of 5-methyldeoxycytosine at CpG-dinucleotide sites throughout the whole genome, particularly in repetitive DNA sequences [4,5]. LINE-1 is one type of repetitive sequence that is dispersed throughout approximately 17% of the entire genome [6,7]. Consequently, LINE-1 methylation is widely considered to comprise the significant proportion of genome-wide methylation [2,8,9]. LINE-1 hypomethylation is correlated with genetic changes during carcinogenesis, including the following: genomic instability [10–14], hypermethylation and mutation of tumour-suppressor genes [12,15], alternate transcription of oncogenes [16] and the deregulation of cancer genes [17,18]. Therefore, many studies have hypothesised that LINE-1 methylation is a potentially useful tool as a universal tumour marker for the detection of cancer DNA [8].

Currently, LINE-1 methylation levels are mostly studied by comparing DNA from tumour tissues to DNA from histologically normal tissues of the same original cell type. However, the processes used to obtain these tissues are difficult and invasive. On the contrary, some studies have used blood samples, which are capable of reflecting the genetic and epigenetic alterations in primary tissues [19–21]; obtaining blood samples is also a simple, gentle and noninvasive procedure. Therefore, blood samples may be a suitable "surrogate" specimen for assessing cancer susceptibility. Moreover, lower LINE-1 methylation levels in blood samples have been correlated with increased risks for various types of cancer such as the following: head and neck cancer, oral cancer [22,23], gastric cancer [24] and bladder cancer [25,28]; on the other hand, LINE-1 methylation levels are observed to be higher in renal cell carcinomas [26].

LINE-1 methylation has been shown to have a different level in each locus of genome [27]. For this reason, we classified the methylation statuses of LINE-1 loci using Combined Bisulfite Restriction Analysis (COBRA) to determine the methylation pattern of the 2 CpG dinucleotides in each LINE-1 sequence [23]. This technique differentiated LINE-

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1 sequences into 4 methylation-status categories: hypermethylated, hypomethylated, and 2 forms of partially methylated loci [23]. We also show that, unlike the case with overall LINE-1 methylation levels, the percentage of hypomethylated loci (%$^{u}C^{u}C$) can be used to significantly distinguish between normal PBMCs and PBMCs from oral squamous cell carcinoma patients [23]. In the present study, we extended our analyses of LINE-1 methylation levels and patterns to PBMCs from patients with six different types of cancer, including cancers of nasopharynx, breast, liver, bile duct, lung and colon.

2. Materials and methods

2.1. Recruited subjects

Two hundred and sixteen cancer patients and 144 normal controls were enrolled in this study. All cancer patients were admitted to the National Cancer Institute of Thailand; this group was composed of patients with pathologically diagnosed cancers of six types: the invasive ductal carcinoma of breast cancer, the adenocarcinoma of colorectal cancer, cholangiocarcinoma, hepatocellular carcinoma, the squamous cell carcinoma of lung cancer and nasopharyngeal cancer. Each cancer type was represented by an equal number of patients (n = 36). The demographic data and clinical stages of the patients were determined using the patient's charts (Table 1).

As a normal control group, cancer-free subjects were randomly selected from the urban area of Bangkok; the normal control group consisted of 72 women and 72 men (mean age ± SD = 48.67 ± 12.10 years). They were asked to complete questionnaires concerning their medical and family histories to confirm that they were free of cancer. This study was approved by the Ethics Committee of the National Cancer Institute of Thailand.

2.2. DNA extraction and bisulfite modification

Heparinised blood samples were collected. Following fractionation of the samples into plasma, buffy-coat and erythrocyte, DNA was extracted from peripheral-blood buffy coats using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). A total of 1 μg DNA was used in the bisulfite treatments. Bisulfite modification of genomic DNA was performed according to a previously published method [23]. Briefly, 1 μg DNA was dissolved in 50 μl distilled water and then denatured in 5.5 μl of 2 M NaOH for 10–30 min at 37 °C. Next, 30 μl of freshly prepared 10 mM hydroquinone (Sigma-Aldrich, Singapore) and 520 μl of 3 M sodium bisulfite (pH 5.0) were added and the solutions were mixed, followed by incubation at 50 °C for 16 h. The bisulfite-treated DNA was recovered using the Wizard DNA Clean-Up Kit (Promega, Madison, WI) according to the manufacturer's protocol. The DNA was then eluted with 50 μl of warm water and desulfonated with 5.5 μl of 3 M NaOH for 5 min. Following elution, the DNA was precipitated with sodium acetate and ethanol (according to standard procedures) using glycogen as a carrier, and the pellets were resuspended in 20 μl of water. The bisulfite-treated DNA samples were stored at −20 °C until use.

Table 1
Demographic data from normal controls and cancer patients.

	Total (n)	Male (n)	Female (n)	Age (mean ± S.D.)
Normal (male + female)	144	72	72	48.67 ± 12.10
Normal (male)	72	72	0	49.63 ± 14.32
Normal (female)	72	0	72	47.72 ± 9.39
Breast cancer	36	0	36	50.28 ± 8.64
Colorectal cancer	36	26	10	59.47 ± 9.30
Cholangiocarcinoma	36	27	9	58.17 ± 7.74
Hepatoma	36	22	14	55.67 ± 10.36
Lung cancer	36	29	7	52.50 ± 12.77
Nasopharyngeal carcinoma	36	32	4	48.19 ± 12.29
All six cancer types	216	136	80	54.03 ± 11.04

2.3. COBRA-LINE-1

Polymerase chain reactions (PCRs) were performed as described elsewhere [9,23]. Primers were designed to be specific to nucleotides in the regulatory region of the LINE-1 sequence (GenBank: M80343). The bisulphite-treated DNA samples were subjected to 40 cycles of amplification using the LINE-1-F (5′-CCGTAAGGGGTTAGGGAGTTTTT-3′) and LINE-1-R (5′-RTAAAACCCTCCRAACCAAATATAAA-3′) primers at an annealing temperature of 50 °C. Next, the LINE-1 amplicons (160 bp in length) were digested with the *TaqI* and *TasI* restriction enzymes in NEB buffer 3 (New England Biolabs, Ontario, Canada) at 65 °C overnight. The PCR-digested products were then run on a non-denaturing 8% polyacrylamide gel. Subsequently, the gel was stained using the SYBR green nucleic-acid stain. All specimens were assayed in duplicate. DNA templates from HeLa, Jurkat and Daudi cell lines were used as positive controls in each experiment as well as for interassay-variation normalisation.

2.4. LINE-1 methylation analysis

We classified LINE-1 loci into four groups depending on the methylation status of the 2 CpG dinucleotides in the 5′ and 3′ of the sequence, as detected by COBRA. These four groups were as follows: (1) LINE-1 loci containing 2 unmethylated CpGs ($^{u}C^{u}C$); (2) LINE-1 loci containing 2 methylated CpGs ($^{m}C^{m}C$); (3) LINE-1 loci containing 5′-methylated and 3′-unmethylated CpGs ($^{m}C^{u}C$); and (4) LINE-1 loci containing 5′-unmethylated and 3′-methylated CpGs ($^{u}C^{m}C$) (Fig. 1A, B). LINE-1 methylation levels and the percentage of loci from each class were calculated using the COBRA-digested LINE-1 products. The intensities of the COBRA-LINE-1 bands were measured using a phosphorimager and the ImageQuant Software (Molecular Dynamics, GE Healthcare, Slough, UK). Following enzymatic digestion, the COBRA-LINE-1 amplicons were separated into 5 DNA fragments of differing lengths: 160, 98, 80, 62 and 18 bp. The 18 bp fragment was not used in the following calculations. The methylation state of the 160 bp fragments was $^{m}C^{u}C$. The methylation state of the 98 bp fragments was $^{u}C^{u}C$. The methylation states of the 80 bp fragments were a mixture of $^{m}C^{m}C$ and $^{u}C^{m}C$. Finally, the methylation states of the 62 bp fragments were a mixture of $^{u}C^{u}C$ and $^{u}C^{m}C$ (Fig. 1C). To calculate the number of CpG dinucleotides from the intensity of each band, the intensity of each band was divided by the number of double-stranded bp of DNA sequence as follows: A = 160 bp fragment intensity/160; B = 98 bp fragment intensity/94; C = 80 bp fragment intensity/79; and D = 62 bp fragment intensity/62. Next, LINE-1 methylation levels were calculated according to the following formulas: LINE-1 methylation level percentage $= 100\times(C+A)/(C+A+A+B+D)$; percentage of $^{m}C^{u}C$ loci $(\%^{m}C^{u}C) = 100\times(A)/(((C-D+B)/2)+A+D)$; percentage of $^{u}C^{m}C$ loci $(\%^{u}C^{m}C) = 100\times(D-B)/((C-D+B)/2)+A+D$; percentage of $^{u}C^{u}C$ loci $(\%^{u}C^{u}C) = 100\times B/(((C-D+B)/2)+A+D)$; and percentage of $^{m}C^{m}C$ loci $(\%^{m}C^{m}C) = 100\times((C-D+B)/2)/(((C-D+B)/2)+D+A)$. Fig. 1D shows an example pattern of a number of LINE-1 loci in both normal and cancer cells.

2.5. Statistical analyses

All statistical analyses were performed using SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, USA). An independent sample *t*-test was performed to determine significant differences between LINE-1 methylation levels in PBMCs from cancer patients compared with normal controls. All *p* values are two-sided, and *p* values less than 0.05 were considered to be statistically significant. A receiver-operating characteristic (ROC) curve analysis was performed

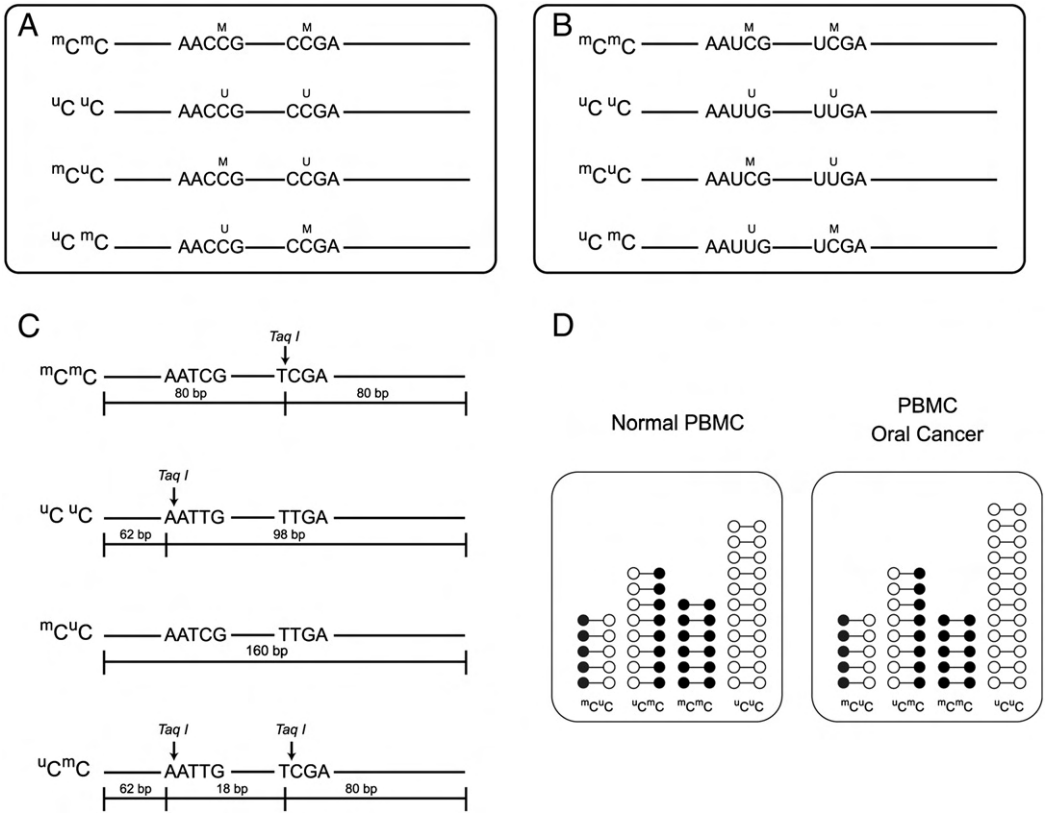

Fig. 1. The methylation patterns of LINE-1 loci detected using COBRA PCR. A: The 5′ UTR of the LINE-1 sequence contains 2 CpG-dinucleotide loci, for which there are 4 possible methylation patterns of the detected LINE-1 sequences (one cell contains 2 alleles). 'M' represents methylated cytosines and 'U' represents unmethylated cytosines in the LINE-1 sequence. COBRA-LINE-1 separated the amplified LINE-1 into 4 products: $^{m}C^{m}C$, $^{u}C^{u}C$, $^{m}C^{u}C$ and $^{u}C^{m}C$. B: Following bisulfite treatment, unmethylated cytosine bases were converted to uracil bases, but the methylated cytosine bases remained unchanged, leading to either loss or retention of CpG-containing restriction-enzyme sites, respectively. C: The PCR products were cut with *TaqI* and *TasI* restriction enzymes. *TaqI*-positive PCR products yielded two 80 bp DNA fragments, while *TasI*-positive fragments yielded 62 and 98 bp fragments. D: A schematic representation comparing the LINE-1 methylation levels between normal PBMCs and PBMCs from oral cancer patients. Hollow circles indicate unmethylated CpGs; dark circles indicate methylated CpGs.

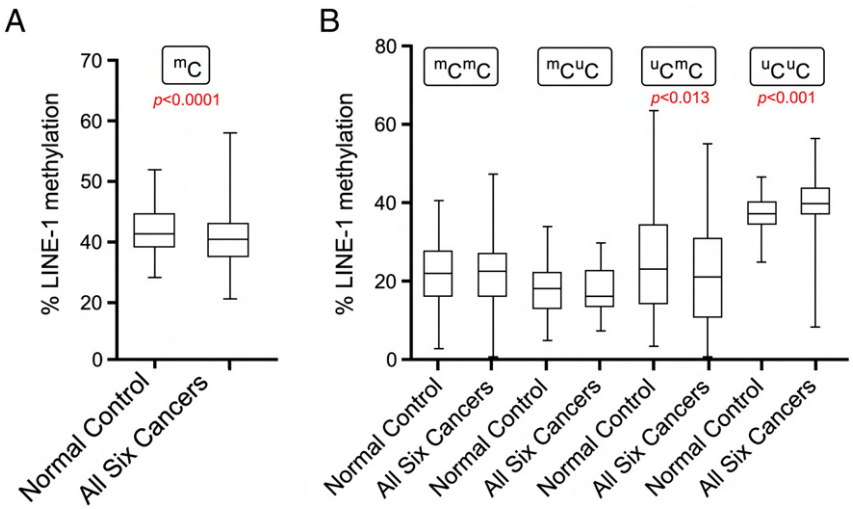

Fig. 2. A comparison of the percentages of the LINE-1 methylation levels in PBMCs from normal controls and all six types of cancer patients. A: The overall LINE-1 methylation. B: Each LINE-1 methylation pattern. Shown are box plots of the LINE-1 methylation levels observed in the patient samples. Black horizontal bars represent the median (IQR). '^{m}C' represents the overall levels of LINE-1 methylation; '$^{m}C^{m}C$' and '$^{u}C^{u}C$' represent hyper- and hypomethylated LINE-1 loci, respectively; and '$^{m}C^{u}C$' and '$^{u}C^{m}C$' represent partially methylated LINE-1 loci.

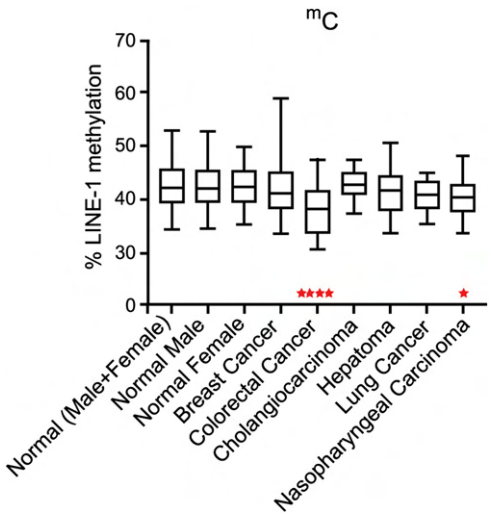

Fig. 3. The percentages of each LINE-1 methylation pattern in PBMCs among the normal controls and the six types of cancer patients. Shown are box plots of the LINE-1 methylation levels observed in the patient samples. Black horizontal bars represent the median (IQR). One asterisk and four asterisks indicate statistical significances of $p = 0.022$, and $p < 0.0001$, respectively.

to verify the ability of COBRA-LINE-1 methylation status to differentiate between the PBMCs of cancer patients and normal controls.

3. Results

3.1. LINE-1 methylation in the PBMCs of cancer patients and normal controls

We analysed LINE-1 methylation levels and patterns in the PBMCs of patients with nasopharynx, breast, liver, bile duct, lung or colon cancers. The LINE-1 methylation level in the normal controls was significantly higher than those observed in all six types of cancer patients ($p < 0.0001$) (Fig. 2A). Among the six cancer types, the LINE-1 methylation levels in PBMCs from patients with colorectal and nasopharyngeal carcinomas were lower ($p < 0.0001$ and $p = 0.022$, respectively) compared with the normal controls (Fig. 3).

3.2. The patterns of LINE-1 methylation in PBMCs from cancer patients and normal controls

Next, we examined the LINE-1 methylation patterns. By comparing the methylation patterns of the normal controls to the patterns of the six types of cancer, the %$^{u}C^{m}C$ and %$^{u}C^{u}C$ patterns were observed to

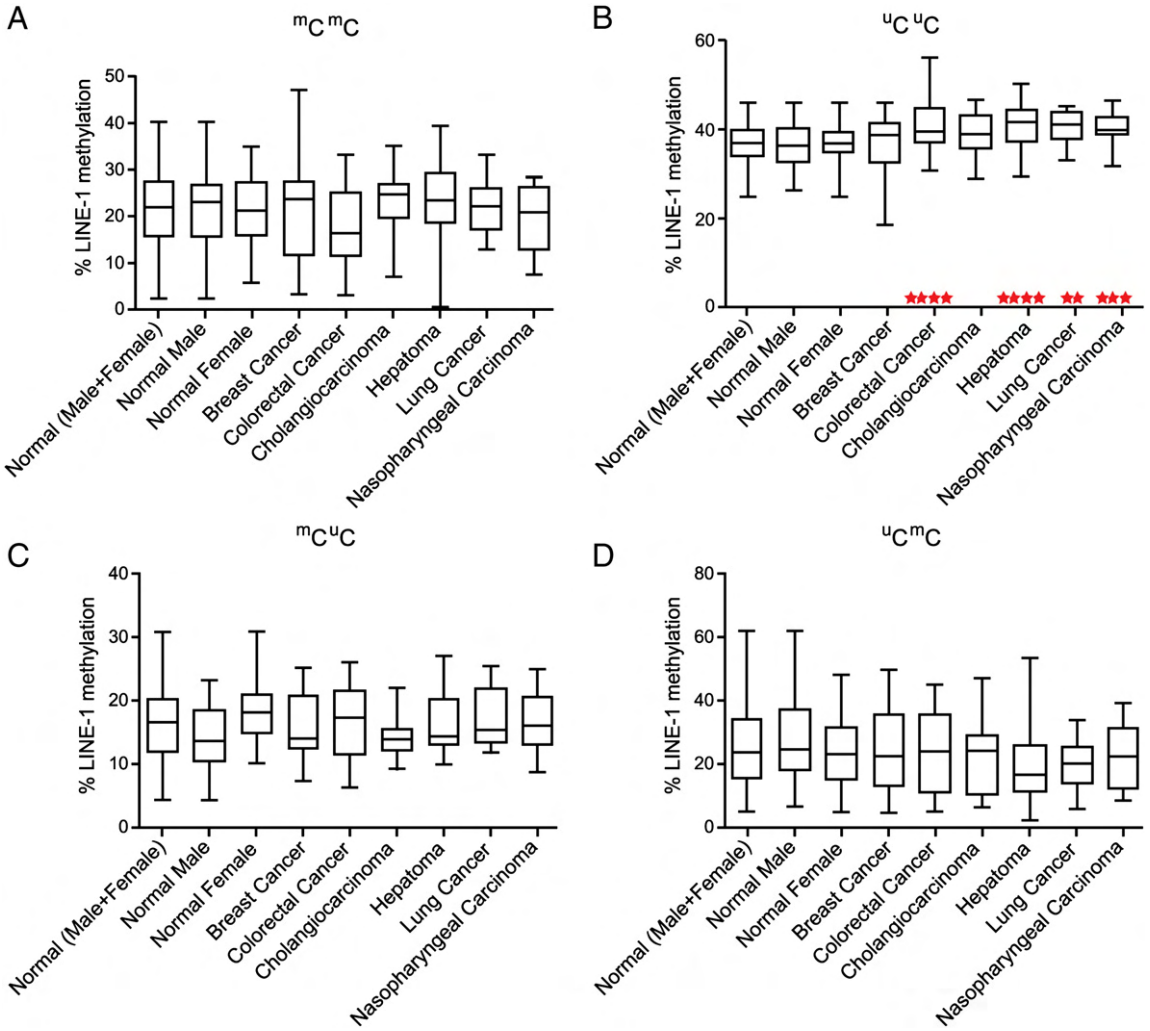

Fig. 4. The percentages of each LINE-1 methylation pattern in PBMCs among the normal controls and the six types of cancer patients. Shown are box plots of the LINE-1 methylation levels observed in the patient samples. Black horizontal bars represent the median (IQR). Two asterisks, three asterisks and four asterisks indicate statistical significances of $p = 0.01$, $p = 0.001$ and $p < 0.0001$, respectively.

be significantly different ($p = 0.013$ and $p < 0.001$, respectively), while the %^{m}C^{m}C and %^{m}C^{u}C patterns were not (Fig. 2B).

In particular, unlike the other patterns, the %^{u}C^{u}C pattern could be used to distinguish normal controls from patients with colorectal cancer ($p < 0.0001$), hepatoma ($p < 0.0001$), lung cancer ($p = 0.01$) and nasopharyngeal carcinoma ($p = 0.001$) (Fig. 4). However, no significant correlations between methylation levels and staging or %^{u}C^{u}C and staging were observed.

3.3. The sensitivity and specificity of LINE-1 methylation as a biomarker of cancer

To evaluate the potential use of LINE-1 analysis as a biomarker, we performed ROC curve analysis. Based on all of our data, we determined several significant tests and we reported the optimal cut-off values, sensitivities, specificities and the areas under the curve (AUCs). As shown in Fig. 5, LINE-1 methylation level (^{m}C) was suitable for detecting colorectal cancer with a cut-off value of 38.32% (AUC = 0.755; sensitivity = 52.78%; specificity = 86.81%). Meanwhile, %^{u}C^{u}C was more effective at detecting cancers of the liver, colon, nasopharynx and lung with cut-off values of 41.15%, 41.40%, 38.60% and 37.15%, respectively (AUCs = 0.755, 0.725, 0.747 and 0.769, respectively; sensitivities = 55.56%, 47.22%, 80.56% and 86.11%, respectively; and specificities = 89.58%, 90.97%, 68.06% and 54.17%, respectively).

4. Discussion

The methylation statuses of the CpG dinucleotides detected using COBRA LINE-1 were in direct agreement with other CpG dinucleotides of LINE-1 sequences [27]. Therefore, we conclude the LINE-1 classes identified using COBRA LINE-1 accurately represent the methylation statuses of the LINE-1 sequences. The patterns ^{u}C^{u}C and ^{m}C^{m}C represent hypomethylated and hypermethylated LINE-1 loci, respectively. COBRA-LINE-1 is also able to measure the percentage of the 2 types of partially methylated LINE-1 loci (^{u}C^{m}C and ^{m}C^{u}C) as well.

The LINE-1 methylation level was less effective at detecting cancer DNA when contaminated with large amounts of normal DNA, particularly from a variety of sources. Our previous report [23] demonstrated that %^{u}C^{u}C was a more specific and sensitive metric than methylation level for detecting oral cancer DNA from an oral rinse [23]. The %^{u}C^{u}C value was also a better metric for differentiating the PBMCs of oral cancer patients from the PBMCs of normal controls. Here, we tested 6 more cancer types. Breast, lung, colon and liver cancers are common worldwide. Nasopharyngeal carcinoma is common in southern China, and cholangiocarcinoma is common in the northern and north-eastern parts of Thailand. The findings of this report are similar to those we published previously [23]; while LINE-1 methylation levels were significantly different in only 2 of the 6 types of cancers tested; %^{u}C^{u}C was able to significantly differentiate four cancer types.

Genome-wide hypomethylation is a molecular feature of cancer tissues, and several studies have reported reduced levels of LINE-1 methylation in primary-cancer tissues and PBMC-derived DNA [8], including samples from head and neck cancer [22,23], bladder cancer [25,28] and gastric cancer [24]. LINE-1 hypermethylation was associated with both an increased risk of renal cancer [26] and a decreased risk of cervical intraepithelial neoplasia [29]. There is still no clear evidence that these changes in methylation are due to interactions between PBMCs and cancer tissues or that these PBMCs are contaminated with circulating cancer cells. Regardless, it will be interesting to further explore the LINE-1 methylation statuses of non tumourous cells from cancer patients.

To date, there have been several reports that measured LINE-methylation levels under several conditions [8]. Here, we show that 2 PBMC-associated cancers are characterised by changes in LINE-1 methylation patterns but not in overall methylation levels. Therefore, we believe that many studies that reported no significant changes in LINE-1 methylation levels likely failed to identify existing cryptic changes in LINE-1 methylation patterns. Because these patterns could alter the biology of these cells [8], we suggest revisiting some earlier "negative" studies [9,30–35] to reanalyse the methylation patterns of LINE-1 loci using COBRA.

In conclusion, we investigated LINE-1 methylation levels and patterns in the PBMCs of six types of cancer. Significant LINE-1 hypomethylation was detected in colon and nasopharyngeal carcinoma compared with controls. When the LINE-1 loci were grouped into four classes based on methylation patterns, distinctive patterns of hypomethylation were revealed in cancers of the colon, liver, lung and nasopharynx. Therefore, we conclude that both the levels and patterns of LINE-1 methylation in PBMCs can be associated with cancer risk but that %^{u}C^{u}C is a more promising biomarker for tumours than overall methylation levels.

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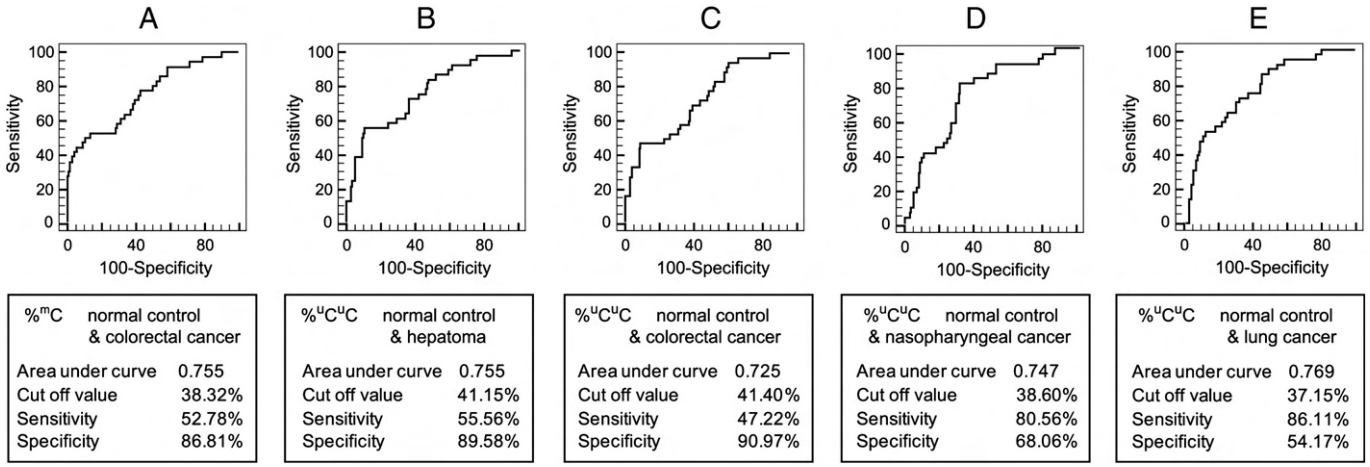

Fig. 5. ROC curve analysis of LINE-1 methylation and cancer detection. (A) The overall level of LINE-1 methylation (^{m}C) and colorectal cancer. (B) The level of ^{u}C^{u}C LINE-1 methylation and hepatoma. (C) The level of ^{u}C^{u}C LINE-1 methylation and colorectal cancer. (D) The level of ^{u}C^{u}C LINE-1 methylation and nasopharyngeal cancer. (E) The level of ^{u}C^{u}C LINE-1 methylation and lung cancer.

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