รายงานวิจัยฉบับสมบูรณ์

โครงการ การควบคุมการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีที่ถูกกระตุ้นโดยเอสโตรเจนในสัตว์ทดลอง

โดย นายปีติ ธุวจิตต์ และคณะ

มิถุนายน พ.ศ. 2555

สัญญาเลขที่ RMU5080055

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คณะผู้วิจัย	สังกัด
1. นายปีติ ธุวจิตต์	ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล
2. นางชนิตรา ธุวจิตต์	ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล
3. นายเอกพจน์ สิงห์สุขสวัสดิ์	ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และสำนักงานคณะกรรมการการอุดมศึกษา

บทคัดย่อ

มะเร็งท่อน้ำดีเป็นมะเร็งชนิดที่มีพยากรณ์โรคไม่ดี เนื่องจากมักจะพบการแพร่กระจายของมะเร็งได้เร็ว โดยที่มะเร็งท่อน้ำดีเป็นมะเร็งที่มีการอุดตันของท่อทางเดินน้ำดี แล้วอาจส่งผลให้เกิดการคั่งของฮอร์โมนเอสโตรเจนได้ โดยที่ฮอร์โมนเอสโตรเจนนี้เป็นสารชีวโมเลกุลที่สามารถส่งผลต่อกิจกรรมของเซลล์มะเร็งได้หลายชนิด อันจะนำไปสู่การพัฒนาของมะเร็งทั้งในด้านการเจริญเติบโตและการแพร่กระจาย กลุ่มผู้วิจัยได้เคยรายงานผลของฮอร์โมนเอสโตรเจนต่อเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี ซึ่งสามารถกระตุ้นการเจริญเติบโตและคุณสมบัติการรุกรานได้ในหลอดทดลอง และระดับฮอร์โมนเอสโตรเจนในซีรั่มของผู้ป่วยมะเร็งท่อน้ำดีเพศชายที่สูงขึ้นและเกี่ยวข้องกับอัตราการรอดชีวิตใน 5 ปีที่ลดลงของผู้ป่วย ในงานวิจัยนี้ ผู้วิจัยได้ทำการทดลองซ้ำเพื่อยืนยันผลการทดลองในหลอดทดลอง ทั้งในรูปแบบของ *in vitro* invasion assay และ wound healing migration assay จากนั้นจึงได้ตรวจสอบการแสดงออกของจีนที่เกี่ยวข้องกับการแพร่กระจายของเซลล์มะเร็งด้วย PCR array assay พบว่าเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีชนิด KKU-M213 มีคุณสมบัติการรุกรานที่เพิ่มขึ้นเมื่อถูกกระตุ้นด้วยเอสโตรเจน และมีการแสดงออกของจีนที่เกี่ยวข้องกับการแพร่กระจายเปลี่ยนไป โดยมีจีนที่แสดงออกเพิ่มขึ้น 11 จีน และแสดงออกลดลง 13 จีน และจากการยืนยันผลการทดลองพบว่า Met น่าจะมีความเกี่ยวข้องกับการกระตุ้นโดยเอสโตรเจน การศึกษาในหนูไร้ขนพบว่า การเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีชนิด KKU-M213 นี้ในหนูที่ให้กินเอสโตรเจนจะสูงกว่าในหนูที่ไม่ได้รับเอสโตรเจน อย่างไรก็ตามในหนูที่ไม่ได้รับเอสโตรเจนพบว่ามีการแพร่กระจายของเซลล์มะเร็งในขณะที่ไม่พบในหนูที่ได้รับเอสโตรเจน ซึ่งแตกต่างจากผลการทดลองที่คาดไว้ ดังนั้นจึงต้องมีการทดลองต่อไปเพื่อยืนยันและหากลไก ผลการทดลองที่ได้น่าจะอธิบายความสำคัญของเอสโตรเจนในผู้ป่วยมะเร็งท่อน้ำดีและแนวทางในการควบคุมการแพร่กระจายของมะเร็งท่อน้ำดีได้ต่อไป

คำสำคัญ : มะเร็งท่อน้ำดี, การรุกราน, การแพร่กระจาย, Met

Abstract

Cholangiocarcinoma (CCA) is a type of poor-prognosis cancer due to its high metastasis rate. According to that CCA is usually found with biliary obstruction, this can be leading to the collection of estrogen. Estrogen is a biomolecule that can effect cancer cell activity and promote cancer progression including the aspect of cell growth and metastasis. We have reported the effect of estrogen to promote growth and invasive properties of CCA cell lines *in vitro*. We also reported the association of increased serum estrogen level and reduction of 5-year survival rate in male CCA patients. In this study, we confirmed the effect of estrogen on invasive property of CCA cell by *in vitro* invasion assay and wound healing migration assay and studied the expression of metastasis genes in CCA cell by PCR array assay. The finding was confirmed the previous study and there were 11 up-regulated and 13 down-regulated metastasis genes found and Met expression has been confirmed its relation to estrogen-induced metastasis. The study in nude mice model was found that tumor growth of KKU-M213 CCA cells was higher in mice treated with estrogen than in non-treated mice. However, the metastasis was found only in non-treated mice while in estrogen-treated mice this was absent, this phenomenon was different to the expected results. So it should be confirmed by the experiment and explored more for the mechanism. The results may explain the importance of estrogen in CCA patients and the way to control CCA matastasis.

Key words : Cholangiocarcinoma, Estrogen, Invasion, Metastasis, Met

หน้าสรุปโครงการ (executive summary)

ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา

1. ชื่อโครงการ (ภาษาไทย) "การควบคุมการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีที่ถูกกระตุ้นโดยเอสโตรเจนในสัตว์ทดลอง"

(ภาษาอังกฤษ) "Control of estrogen-stimulated cholangiocarcinoma cell metastasis in animal model"

2. ชื่อหัวหน้าโครงการ นายปีติ ธุวจิตต์

หน่วยงานที่สังกัด ภาควิชาวิทยาภูมิคุ้มกัน

คณะแพทยศาสตร์ศิริราชพยาบาล

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3. สาขาวิชาที่ทำการวิจัย Molecular biology of cancer

Keywords ของข้อเสนอโครงการ Cholangiocarcinoma, Estrogen, Invasion, Metastasis, Met

5. ระยะเวลาดำเนินการ 3 ปี

6. ได้เสนอโครงการนี้ หรือโครงการที่มีส่วนเหมือนกับเรื่องนี้บางส่วน เพื่อขอทุนต่อแหล่งทุนอื่นที่ใดบ้าง

☐ ไม่ได้เสนอต่อแหล่งทุนอื่น

☑ เสนอต่อ

6.1 ทุนวิจัยอุดหนุนทั่วไป มหาวิทยาลัยขอนแก่น งบประมาณปี 2550

(ภาษาไทย) "บทบาทของเอนไซม์ cyclooxygenase 2 ในกระบวนการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีที่ถูกกระตุ้นด้วยฮอร์โมนเอสโตรเจน "

(ภาษาอังกฤษ) " The role of cyclooxygenase 2 in estrogen-associated metastasis of cholangiocarcinoma "

กำหนดทราบผล (หรือสถานภาพที่ทราบ)

ปิดโครงการแล้ว

6.2 ทุนส่งเสริมกลุ่มนักวิจัยอาชีพ ศูนย์พันธุวิศวกรรมศาสตร์และเทคโนโลยีชีวภาพแห่งชาติ งบประมาณปี 2550

(ภาษาไทย) "แบบแผนการแสดงออกของจีนที่เกี่ยวข้องกับกระบวนการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีซึ่งถูกกระตุ้นด้วยเอสโตรเจนและวิธีการควบคุม"

(ภาษาอังกฤษ) "Genetic profile and control of estrogen-induced metastasis in cholangiocarcinoma cells"

กำหนดทราบผล (หรือสถานภาพที่ทราบ)

อยู่ระหว่างการดำเนินการ

7. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

มะเร็งท่อน้ำดีเป็นมะเร็งที่มีอุบัติการสูงมากในภาคตะวันออกเฉียงเหนือของประเทศไทย โดยที่มะเร็งท่อน้ำดีมีลักษณะเจริญเติบโตช้า (slow progression) และมีการแพร่กระจายที่รวดเร็วมาก (rapid metastasis) ทำให้การรักษาโดยการผ่าตัดทำได้ไม่สมบูรณ์และเป็นสาเหตุหลักที่ทำให้ผู้ป่วยถึงแก่ชีวิต การศึกษาเพื่อให้เข้าใจถึงกลไกการแพร่กระจายของเซลล์มะเร็ง จะเป็นทางนำสู่การป้องกันหรือลดอัตราการแพร่กระจายของมะเร็งได้ และช่วยให้พยากรณ์โรคดีขึ้น

จากผลการทดลองเบื้องต้นของคณะผู้วิจัยพบว่า ระดับของฮอร์โมนเอสโตรเจนในกระแสเลือดของผู้ป่วยมะเร็งท่อน้ำดีเพศชาย มีระดับที่สูงกว่าปกติอย่างมีนัยสำคัญ และสัมพันธ์กับ survival time ที่สั้นของผู้ป่วยเมื่อเทียบกับผู้ป่วยมะเร็งท่อน้ำดีที่มีระดับเอสโตรเจนไม่สูง โดยที่ระดับเอสโตรเจนที่สูงนี้ สัมพันธ์กับภาวะการอุดตันของทางเดินน้ำดี นอกจากนี้ จากผลการทดลองเบื้องต้นของผู้วิจัยยังพบอีกว่า ฮอร์โมนเอสโตรเจนในระดับที่สูงขึ้นของผู้ป่วยนี้ สามารถที่จะกระตุ้นให้เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีให้มีความสามารถในการรุกรานที่มากขึ้น และ

สามารถที่จะยับยั้งกระบวนการนี้ได้ด้วย tamoxifen ซึ่งเป็นยาต้านเอสโตรเจน นอกจากนี้การรุกรานที่มากขึ้นนี้เกิดขึ้นพร้อมกับการแสดงออกที่เพิ่มขึ้นของโปรตีนเทรฟอยด์ชนิด TFF1 ซึ่งเป็นโปรตีนที่ส่งสัญญาณให้เซลล์เคลื่อนที่ได้ และการแสดงออกที่ลดลงของโปรตีน E-cadherin ซึ่งเป็นโปรตีนที่ทำหน้าที่ในการยึดเกาะกันของเซลล์ ดังนั้นจึงเป็นที่น่าสนใจว่าฮอร์โมนเอสโตรเจนนี้จะส่งผลต่อพยากรณ์โรคที่ไม่ดีของผู้ป่วย โดยจะกระตุ้นให้เซลล์มะเร็งเกิดการแพร่กระจายที่มากขึ้น ผ่านการเพิ่มขึ้นของโปรตีนเทรฟอยด์ชนิด TFF1 และการลดลงของโปรตีน E-cadherin กลไกที่ฮอร์โมนเอสโตรเจนไปกระตุ้นเซลล์มะเร็งท่อน้ำดีให้มีการรุกรานที่มากขึ้นนี้ จึงเป็นที่น่าสนใจที่จะศึกษา ซึ่งหากได้ข้อมูลที่ถูกต้องก็จะสามารถพัฒนาไปใช้ในการควบคุมการแพร่กระจายของมะเร็ง ในผู้ป่วยมะเร็งท่อน้ำดีต่อไปได้

การศึกษาในหลอดทดลองเป็นการศึกษาวิจัยที่สามารถควบคุมตัวแปรต่างๆ ได้ง่าย ได้ผลการวิจัยที่ชัดเจนและเชื่อถือได้ อย่างไรก็ตามปัจจัยต่างๆ ทางสรีรวิทยาที่อาจส่งผลต่อความสามารถในการรุกรานของเซลล์มะเร็งท่อน้ำดียังมีอีกมาก ซึ่งไม่สามารถตรวจสอบได้โดยใช้การศึกษาในหลอดทดลองเพียงอย่างเดียว ดังนั้นการศึกษาวิจัยจึงต้องเปลี่ยนไปทำในสัตว์ทดลอง ซึ่งสามารถศึกษาในสภาพที่มีปัจจัยทางสรีรวิทยามาเกี่ยวข้องได้ ขณะเดียวกันก็สามารถควบคุมปัจจัยต่างๆ ได้ดีกว่าการศึกษาในตัวผู้ป่วย ดังนั้น ผู้วิจัยจึงจะใช้การศึกษาในสัตว์ทดลองในโครงการวิจัยนี้ เพื่อยืนยันผลการทดลองที่ผ่านมาและศึกษากลไกที่เอสโตรเจนกระตุ้นเซลล์มะเร็งท่อน้ำดีให้ละเอียดยิ่งขึ้น

8. วัตถุประสงค์

1. เพื่อศึกษากลไกการกระตุ้นการแพร่กระจายของมะเร็งท่อน้ำดีโดยฮอร์โมนเอสโตรเจน
2. เพื่อหาแนวทางการศึกษาการแพร่กระจายของมะเร็งในสัตว์ทดลอง
3. เพื่อศึกษาแนวทางการควบคุมการแพร่กระจายของมะเร็งท่อน้ำดี

9. ระเบียบวิธีวิจัย

โครงการวิจัยนี้แบ่งเป็น 2 ระยะ ดังนี้

ระยะที่ 1 เป็นการศึกษาในหลอดทดลอง และเตรียมเซลล์มะเร็งท่อน้ำดีที่ติดฉลากด้วย green fluorescence protein ใช้เวลา 1 ปี

ตัวอย่างที่ใช้ศึกษา

เซลล์มะเร็งท่อน้ำดี KKU-M213 ที่พัฒนาจากเซลล์มะเร็งของผู้ป่วยมะเร็งท่อน้ำดีที่เข้ามารับการรักษา ณ โรงพยาบาลศรีนครินทร์ ผ่านการเพาะเลี้ยงโดยหน่วยปฏิบัติการวิจัยพยาธิวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

วิธีการวิจัย

ทำการเพาะเลี้ยงเซลล์มะเร็ง KKU-M213 และทำการ transfect จีนของ green fluorescence protein เข้าไปในพาหะ แล้วนำเข้าเซลล์

ทำการเพาะเลี้ยงเซลล์ต่อไปจนได้เซลล์ปริมาณมากพอ แล้วจึงทำการตรวจสอบการรุกรานด้วยเทคนิค *in vitro* invasion assay และ wound healing migration assay และศึกษาการแสดงออกของจีนที่เกี่ยวข้องกับการแพร่กระจาย โดยใช้เทคนิค PCR array และยืนยันผลด้วย real time RT-PCR

ระยะที่ 2 เป็นการปลูกถ่ายเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีที่ทำการ transfect plasmid เข้าสู่หนู nude mice และวัดคุณสมบัติต่างๆ ของเซลล์ โดยเฉพาะคุณสมบัติการแพร่กระจาย ใช้เวลา 2 ปี

ตัวอย่างที่ใช้ศึกษา

Nude mice ซึ่งจะต้องผ่านการรับรองทางจริยธรรมจากสถาบันต้นสังกัด

วิธีการวิจัย

ทำการเลี้ยงหนูด้วยวิธีมาตรฐานและปราศจากเชื้อ จากนั้นทำการปลูกถ่ายเซลล์มะเร็งเข้าไป จากนั้นเลี้ยงหนูต่อไปโดยฉีดหรือให้กิน 17β-estradiol (ชนิดที่ละลายน้ำ) เลี้ยงต่อไปอีกแล้วทำการวัดการแพร่กระจายด้วยเครื่อง fluoro-imaging จากนั้นจึงฆ่าหนู และตรวจสอบการแพร่กระจายของเซลล์มะเร็งโดยใช้ green fluorescence protein เป็นตัวตรวจวัด ทำการวัดขนาด, น้ำหนัก และเก็บชิ้นเนื้อมะเร็งในทุกตำแหน่งที่มีการแพร่กระจาย และวัดการแสดงออกของจีนที่สนใจจากระยะแรกด้วยเทคนิค real time RT-PCR และ immunohistochemistry

ทำการทดลองยับยั้งการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีที่ปลูกถ่ายในหนู nude mice โดยใช้ยาต่างๆ ได้แก่ tamoxifen, celecoxib เป็นต้น

เขียน manuscript 2 ฉบับ ว่าด้วยการศึกษาจีนที่เกี่ยวข้องกับการแพร่กระจายของมะเร็งท่อน้ำดีที่ถูกกระตุ้นด้วยเอสโตรเจน และเรื่องของการทดลองใน nude mice

10. จำนวนโครงการที่ผู้สมัครกำลังดำเนินการอยู่ 3 โครงการ

1. ชื่อโครงการ Genetic profile and control of estrogen-induced metastasis in cholangiocarcinoma cells **หัวหน้าโครงการ**

ผู้ช่วยศาสตราจารย์ ดร. นพ. ปีติ ธุวจิตต์

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แหล่งทุนที่ให้การสนับสนุน ทุนส่งเสริมกลุ่มนักวิจัยอาชีพ สวทช.

งบประมาณที่ได้รับ 600,000 บาท

สถานะผู้สมัคร ☑ หัวหน้าโครงการ

☐ ผู้ร่วมโครงการ

เวลาที่ใช้ทำโครงการวิจัยในโครงการเป็น 8 ชั่วโมงต่อสัปดาห์

2. ชื่อโครงการ ตัวตรวจวัดระดับโมเลกุลในตัวอย่างทางคลินิกจากการส่องกล้องท่อน้ำดีในผู้ป่วยที่มีภาวะท่อน้ำดีตีบตัน: เป้าหมายในการวินิจฉัย พยากรณ์โรคและการรักษา

หัวหน้าโครงการ

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สถานะผู้สมัคร ☑ หัวหน้าโครงการ

☐ ผู้ร่วมโครงการ

เวลาที่ใช้ทำโครงการวิจัยในโครงการเป็น 8 ชั่วโมงต่อสัปดาห์

3. ชื่อโครงการ การศึกษาบทบาทของตัวรับเอสโตรเจนชนิด GPER ในเซลล์เพาะเลี้ยงมะเร็งเต้านมกับการตอบสนองต่อเคมีบำบัด และการแสดงออกในชิ้นเนื้อมะเร็งเต้านมร่วมกับความสัมพันธ์ต่อการแสดงออกทางคลินิก

หัวหน้าโครงการ

ผู้ช่วยศาสตราจารย์ ดร. นพ. ปีติ ธุวจิตต์

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งบประมาณที่ได้รับ 1,000,000 บาท

สถานะผู้สมัคร ☑ หัวหน้าโครงการ

☐ ผู้ร่วมโครงการ

เวลาที่ใช้ทำโครงการวิจัยในโครงการเป็น 8 ชั่วโมงต่อสัปดาห์

11. ความเชื่อมโยงกับต่างประเทศ

กลุ่มผู้วิจัยได้ติดต่อกับ Dr. Sue Eccels จาก The Institute of Cancer Research: Royal Cancer Hospital, 15 Cotswold Rd, Belmont, Sutton Surrey, London, United Kingdom ซึ่งเป็นผู้เชี่ยวชาญการศึกษาการแพร่กระจายของมะเร็งในสัตว์ทดลอง เพื่อที่จะส่งนักศึกษาคือนายเอกพจน์ สิงห์สุขสวัสดิ์ไปทำงานวิจัยต่อไป

กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับทุนสนับสนุนจาก ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา และทุนส่งเสริมกลุ่มนักวิจัยอาชีพ สวทช. นายเอกพจน์ สิงห์สุขสวัสดิ์ ได้รับทุนสนับสนุนการทำงานส่วนหนึ่งจาก ทุนส่งเสริมกลุ่มนักวิจัยอาชีพ สวทช. โดย ศาสตราจารย์ ดร.โสพิศ วงศ์คำ ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี ได้รับการอนุเคราะห์จากรองศาสตราจารย์บรรจบ ศรีภา ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น การติดฉลากเซลล์มะเร็งท่อน้ำดี ได้รับการอนุเคราะห์จากดร.บรรพต ศิริเดชาดิลก หน่วยอณูชีววิทยาการแพทย์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล การศึกษาในชิ้นเนื้อได้รับการอนุเคราะห์จาก อ. นพ. คมกริช จ่างแก้ว ภาควิชาพยาธิวิทยา แพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

สุดท้ายนี้ ผู้วิจัยต้องขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย ที่ได้ให้โอกาสในการทำวิจัยโครงการนี้

สารบัญ

เนื้อหางานวิจัย

ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา

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1. ชื่อโครงการ (ภาษาไทย) "การควบคุมการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีที่ถูกกระตุ้นโดยเอสโตรเจนในสัตว์ทดลอง"

(ภาษาอังกฤษ) "Control of estrogen-stimulated cholangiocarcinoma cell metastasis in animal model"

2. คณะผู้วิจัย

2.1) หัวหน้าโครงการวิจัย นายปีติ ธุวจิตต์ Peti Thuwajit

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2.2) ผู้ร่วมวิจัย นางชนิตรา ธุวจิตต์ Chanitra Thuwajit

2.3) ผู้ร่วมวิจัย นายเอกพจน์ สิงห์สุขสวัสดิ์ Ekapot Singsuksawat

3. Introduction

Cholangiocarcinoma (CCA) is the cancer of bile duct epithelial cell. Worldwide, CCA is rare but the incidence is increasing.[1] In contrast, CCA is the endemic carcinoma with causes serious public health problems in north-eastern part of Thailand. In this area, its etiology is associated with a liver fluke *Opisthorchis viverrini* infection.[2] CCA is one of the worst prognosis carcinoma while the median survival rate is low and patients should die within a year of diagnosis.[3] The poor prognosis of CCA is due to its high metastasis character. In general, metastasis is a hallmark of cancer cells seeding at distance organ and this process can be promoted by various biological molecules including hormone such as estrogen. Estrogen is a well-known biological substance which can stimulate the progression of various type of cancer especially in female carcinoma, such as breast, endometrial and ovarian cancer.[4-6] *In vitro* studies have shown the induction of invasion by estrogen in some cancer such as thyroid, prostate and osteosarcoma.[7-9] The study in mouse xenograph model also showed the promotion of lymph node metastasis in ovarian cancer cell.[6] In addition estrogen has been reported as an agent that can induce expression of a set of metastasis genes.[9] Previous study showed that CCA had impairment of estrogen metabolizing enzyme 17β-hydroxysteroid dehydrogenase type 2 that could lead to the accumulation of estrogen in plasma.[10] Moreover, our recent report showed that estrogen is accumulated in CCA patients' sera and associated with short survival time after surgery.[11]

Recently, estrogens have been shown to target the biliary tree, by modulate the secretory function of interleukin-6 (IL-6) in non-neoplastic cholangiocytes.[12] In CCA,

there were several evidences showed that estrogen could have the effect on cancer cell. Estrogen could induce cell proliferation in CCA cell lines.[12-14] Moreover, estrogen could stimulate secretory function of some active proteins such as IL-6 and vascular endothelial growth factor (VEGF) in CCA cell lines.[12,13] *In vivo* study, it has been a report of increasing of estrogen in CCA tissue using immunohistochemistry method.[15] In addition of the function of estrogen, while estrogen receptors (ER) were reported minimally expressed in normal bile duct, it has been presented the expression of ER in CCA tissue.[16] These evidences suggested that estrogen could have effect on CCA cell. *In vitro* study from our report also showed that estrogen could promote the proliferation and invasion of KKU-100 and KKU-M213 CCA cells.[11] So it is interesting that these effects of estrogen on CCA cells should be proven *in vivo* such as using animal model.

One of the effector products which can be responded to estrogen stimulation and has potential to stimulate cell migration is trefoil factor family 1 (TFF1). TFF1 is a member of a trefoil factor family including TFF2 and TFF3.[17] All of trefoil proteins have ability to stimulate epithelial cell migration and play roles in protection against damage of mucosa by promote wound healing especially along gastrointestinal tract.[18] TFF1 is expressed majorly in stomach mucosa but not expressed in hepatocytes.[19] While TFF1 is minimally expressed in normal large bile duct, it has been reported the high expression of TFF1 in CCA.[19,20] The *in vitro* studies have shown the stimulation of TFF1 on cancer cells invasion in breast cancer and CCA.[20,21] Genes of all trefoil factor family members are located as cluster on chromosome 21q22.3, and for TFF1 gene, it contains estrogen responsive element which could be promoted transcription by estrogen.[22] So TFF1 is suspected as a metastasis promoting agent in cancer due to its

motogenic property and it could be stimulated by estrogen. Our recent report showed the association of *TFF1* gene expression and estrogen-induced invasion of KKU-100 and KKU-M213 CCA cells *in vitro*.[11] These evidences support the possibility that estrogen could induce invasive property and metastasis of CCA in associate with TFF1 trefoil protein. Unfortunately the expression of *TFF1* gene was disappear in both KKU-100 and KKU-M213 CCA cells and could not be detected in each CCA cell lines so we could not prove this hypothesis in *in vivo* model.

In this study we tried to set up the animal model of estrogen regulated CCA cells. KKU-M213 CCA cells were selected for this study because they have more responsible to estrogen than other cells. CCA cells should be re-checked their response to estrogen and the expression of metastasis associated genes after estrogen treatment. KKU-M213 CCA cell should be also labeled with fluorescence such as green fluorescent protein by gene transfection technique. After that the labeled cells would be implanted into animal, nude mouse is suitable, and measured the growth and metastasis of CCA cells. The manipulation by tamoxifen should be performed. The metastasis genes expression in *in vivo* system should be evaluated too and selected genes should be manipulated to check their importance in estrogen induced CCA progression and the way to control.

4. Objectives

1. To determine the mechanism of estrogen induced metastasis in cholangiocarcinoma
2. To establish the animal model for studying the metastasis process of cancer
3. To identify the method for controlling the metastasis of cholangiocarcinoma

5. Materials and methods

1. Cholangiocarcinoma cell line

KKU-M213 CCA cell line was cultured in HAM-F12 media with 10% fetal bovine serum (FBS) supplement, antibiotics and antifungal including 0.1 U/ml penicillin G sodium, 0.1 mg/ml streptomycin and 5mg/ml of amphotericin B to prevent microbial contaminations, in 5% CO_2 and 37^{o}C humidified incubator. Cells were cultured for 2 days then the media would be changed. The passaging procedure would be done when cell reached 100% confluency.

2. Metastasis PCR array

By the hypothesis that estrogen could induce CCA cell invasion and metastasis, the metastasis PCR array was performed for comparing of metastasis gene expression in KKU-M213 CCA cell which treated or not treated with estrogen. Briefly, approximately 2x10^{6} cells of KKU-M213 CCA cell would be cultured in 25 cm^{2} flasks. Cells would be cultured in phenol red-free DMEM with 10% FBS, 0.1 U/ml penicillin G sodium, 0.1 mg/ml streptomycin and 5mg/ml of amphotericin B for 48 hours and then treated with 0 or 1 nM 17β-estradiol for other 48 hours. After that, cells would be harvested and RNA extraction would be performed using 5-Prime® PerfectPure RNA Tissue Kit (5-Prime). RNA concentration would be determined by Nanodrop® 1000 (Thermo Scientific). RNA would be converted to cDNA by SABiosciences NEW RT2 PCR Array First Strand Kit®. The metastasis PCR array was performed using Superarray Human Tumor Metastasis RT² Profiler PCR® Arrays, Type G (384-well)

(SAbioscience) and SABiosciences RT² qPCR SYBR Green Master Mix®. Gene list in this kit was shown in table 1. Real time PCR was performed by Light cycler 480™ real time PCR machine (Roche) and the cycle threshold (Ct) values were used to calculate for the comparison of gene expressions by $2^{-\Delta\Delta Ct}$ formula.

Table 1 Metastasis genes and internal control from Superarray Human Tumor Metastasis RT² Profiler PCR® Arrays with expected change for increasing of invasion and metastasis

	Unigene	**GeneBank**	**Symbol**	**Description**	**Change of expression that could be leading to increase invasion and metastasis**
1	Hs.158932	NM_000038	APC	Adenomatous polyposis coli	Down
2	Hs.100426	NM_015399	BRMS1	Breast cancer metastasis suppressor 1	Down
3	Hs.251526	NM_006273	CCL7	Chemokine (C-C motif) ligand 7	Up
4	Hs.502328	NM_000610	CD44	CD44 molecule (Indian blood group)	Up

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
5	Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Down
6	Hs.116471	NM_001797	CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	Down
7	Hs.171054	NM_004932	CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)	Down
8	Hs.512599	NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	Down
9	Hs.162233	NM_001273	CHD4	Chromodomain helicase DNA binding protein 4	Down
10	Hs.508716	NM_001846	COL4A2	Collagen, type IV, alpha 2	Down
11	Hs.143212	NM_003650	CST7	Cystatin F (leukocystatin)	Down
12	Hs.208597	NM_001328	CTBP1	C-terminal binding protein 1	Down
13	Hs.445981	NM_001903	CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa	Down

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
14	Hs.632466	NM_000396	CTSK	Cathepsin K	Up
15	Hs.418123	NM_001912	CTSL1	Cathepsin L1	Up
16	Hs.522891	NM_000609	CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	Up
17	Hs.593413	NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4	Up
18	Hs.22393	NM_003677	DENR	Density-regulated protein	Up
19	Hs.523329	NM_004442	EPHB2	EPH receptor B2	Down
20	Hs.434059	NM_001986	ETV4	Ets variant gene 4 (E1A enhancer binding protein, E1AF)	Up
21	Hs.374477	NM_005243	EWSR1	Ewing sarcoma breakpoint region 1	Up
22	Hs.481371	NM_005245	FAT	FAT tumor suppressor homolog 1 (Drosophila)	Down
23	Hs.165950	NM_002011	FGFR4	Fibroblast growth factor receptor 4	Up

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
24	Hs.646917	NM_002020	FLT4	Fms-related tyrosine kinase 4	Up
25	Hs.203717	NM_002026	FN1	Fibronectin 1	Up
26	Hs.333418	NM_014164	FXYD5	FXYD domain containing ion transport regulator 5	Down
27	Hs.82963	NM_000825	GNRH1	Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	Down
28	Hs.208229	NM_032551	KISS1R	KISS1 receptor	Down
29	Hs.396530	NM_000601	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	Up
30	Hs.44227	NM_006665	HPSE	Heparanase	Up
31	Hs.37003	NM_005343	HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	Up
32	Hs.90753	NM_006410	HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	Down

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
33	Hs.160562	NM_000618	IGF1	Insulin-like growth factor 1 (somatomedin C)	Up
34	Hs.83077	NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)	Up
35	Hs.126256	NM_000576	IL1B	Interleukin 1, beta	Up
36	Hs.846	NM_001557	IL8RB	Interleukin 8 receptor, beta	Up
37	Hs.524484	NM_002206	ITGA7	Integrin, alpha 7	Down
38	Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	Up
39	Hs.527778	NM_002231	CD82	CD82 molecule	Down
40	Hs.95008	NM_002256	KISS1	KiSS-1 metastasis-suppressor	Down
41	Hs.505033	NM_004985	KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Up
42	Hs.356262	NM_002295	RPSA	Ribosomal protein SA	Up

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
43	Hs.599039	NM_006500	MCAM	Melanoma cell adhesion molecule	Down
44	Hs.567303	NM_002392	MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	Up
45	Hs.132966	NM_000245	MET	Met proto-oncogene (hepatocyte growth factor receptor)	Up
46	Hs.444986	NM_006838	METAP2	Methionyl aminopeptidase 2	Up
47	Hs.651869	NM_002410	MGAT5	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase	Up
48	Hs.2258	NM_002425	MMP10	Matrix metallopeptidase 10 (stromelysin 2)	Up
49	Hs.143751	NM_005940	MMP11	Matrix metallopeptidase 11 (stromelysin 3)	Up

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
50	Hs.2936	NM_002427	MMP13	Matrix metallopeptidase 13 (collagenase 3)	Up
51	Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Up
52	Hs.375129	NM_002422	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	Up
53	Hs.2256	NM_002423	MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	Up
54	Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Up
55	Hs.525629	NM_004689	MTA1	Metastasis associated 1	Up
56	Hs.336994	NM_014751	MTSS1	Metastasis suppressor 1	Down

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
57	Hs.202453	NM_002467	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	Up
58	Hs.437922	NM_005376	MYCL1	V-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	Up
59	Hs.187898	NM_000268	NF2	Neurofibromin 2 (merlin)	Down
60	Hs.118638	NM_000269	NME1	Non-metastatic cells 1, protein (NM23A) expressed in	Down
61	Hs.463456	NM_002512	NME2	Non-metastatic cells 2, protein (NM23B) expressed in	Down
62	Hs.9235	NM_005009	NME4	Non-metastatic cells 4, protein expressed in	Down
63	Hs.279522	NM_006981	NR4A3	Nuclear receptor subfamily 4, group A, member 3	Up

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
64	Hs.466871	NM_002659	PLAUR	Plasminogen activator, urokinase receptor	Up
65	Hs.409965	NM_002687	PNN	Pinin, desmosome associated protein	Up
66	Hs.500466	NM_000314	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	Down
67	Hs.408528	NM_000321	RB1	Retinoblastoma 1 (including osteosarcoma)	Down
68	Hs.494178	NM_006914	RORB	RAR-related orphan receptor B	Down
69	Hs.436687	NM_003011	SET	SET translocation (myeloid leukemia-associated)	Up
70	Hs.12253	NM_005901	SMAD2	SMAD family member 2	Up
71	Hs.75862	NM_005359	SMAD4	SMAD family member 4	Up

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
72	Hs.195659	NM_005417	SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	Up
73	Hs.514451	NM_001050	SSTR2	Somatostatin receptor 2	Up
74	Hs.371720	NM_003177	SYK	Spleen tyrosine kinase	Up
75	Hs.475018	NM_005650	TCF20	Transcription factor 20 (AR1)	Up
76	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1	Up
77	Hs.633514	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2	Down
78	Hs.701968	NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	Down
79	Hs.591665	NM_003256	TIMP4	TIMP metallopeptidase inhibitor 4	Down

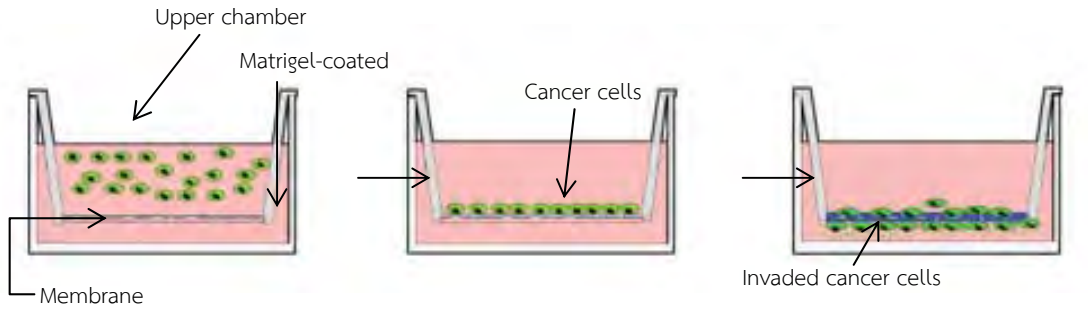
	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
80	Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	Down
81	Hs.654481	NM_000546	TP53	Tumor protein p53	Up
82	Hs.155942	NM_002420	TRPM1	Transient receptor potential cation channel, subfamily M, member 1	Down
83	Hs.160411	NM_000369	TSHR	Thyroid stimulating hormone receptor	Up
84	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A	Up
85			B2M	β2-microglobulin	Internal control
86			HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	Internal control
87			RPL13A	Ribosomal protein L13A	Internal control
88			GAPDH	Glyceroldehyde-3-phosphate dehydrogenase	Internal control

	Unigene	GeneBank	Symbol	Description	Change of expression that could be leading to increase invasion and metastasis
89			ACTB	β-actin	Internal control

3. Invasion assay

To confirm that KKU-M213 CCA cells could be manipulated their invasive property by estrogen and tamoxifen, *in vitro* invasion assay should be performed. Briefly, approximately 10^5 cells of KKU-M213 CCA cell would be cultured in 6-well cell culture plate. After 24 hours the media would be changed to phenol red-free DMEM with 10% FBS, 0.1 U/ml penicillin G sodium, 0.1 mg/ml streptomycin and 5mg/ml of amphotericin B and continued for 48 hours. After that, cells would be treated with 0, 1 or 10 nM 17β-estradiol with or without 10 μM tamoxifen for more 48 hours. Then cells would be harvested and the aliquots of $4x10^4$ cells were plated in upper chamber of 24-well formatted BD Biocoat Matrigel® Invasion Chambers, 8μm pore size (Becton-Dickinson) (Figure 1) in the same media (0, 1 or 10 nM 17β-estradiol with or without 10 μM tamoxifen) and incubated in CO_2 incubator at 37^oC. Cancer cells would invade through matrigel and move across the membrane via pore and then attach the lower part of the membrane. After 28-hour incubation time, the upper chambers were taken and matrigel with non-invaded cells in upper chamber was removed. Cancer cells that attached with the lower part of membrane, marked as invaded cells, would be fixed with 5% paraformaldehyde and stained with crystal violet. Total invaded cell counting would be

performed under inverted microscope and the ratios of invaded cell among the treatment conditions were calculated.



Modified from http://www.bdbiosciences.com/eu/cellculture/endothelialcells/index.jsp

Figure 1 Diagram of Matrigel® coated transwell *in vitro* invasion assay. After incubated for 28 hours upper chamber from C should be scraped the upper part of membrane for removing the non-invaded cells. The upper chambers would be stained for the remaining invaded cell that attached at the lower part of the membrane.

4. Migration assay

To confirm that KKU-M213 CCA cells could be manipulated their migratory property by estrogen and tamoxifen, wound healing assay should be performed. Briefly, approximately $5x10^5$ cells of KKU-M213 CCA cell would be cultured in 6-well cell culture plate. After 24 hours the media would be changed to phenol red-free DMEM-F12 with 10% FBS, 0.1 U/ml penicillin G sodium, 0.1 mg/ml streptomycin and 5mg/ml of amphotericin B and continued for 48 hours. A scratch with pipette tip would be done in each well and then cells would be treated with 0 or 1 nM 17β-estradiol with or without 10 μM tamoxifen for more 48 hours. During this period, cells were photographed and distance between migration front at time 0, 3, 6, 9, 12 and 24 hour were measured. The migration of cancer cells in each condition would be compared. Cells were harvested and prepared for RNA extraction for validation of gene expression by real time RT-PCR.

5. Validation of gene expression by real time RT-PCR

MET and TIMP4 were the first 2 genes selected for validation by real time RT-PCR of their expression in estrogen and tamoxifen treated KKU-M213 CCA cells. Cells after migration assay were extracted their RNA 5-Prime® PerfectPure RNA Tissue Kit. RNA concentration would be determined by Nanodrop® 1000. RNA would be converted to cDNA by Superscript® III First-Strand Synthesis System (Invitrogen). Internal control used in this experiment was 36B4 ribosomal protein. Real time PCR was performed using LightCycler® 480 SYBR Green I Master (Roche) by Light cycler 480™ real time PCR machine and the cycle threshold (Ct) values were used to calculate for the

comparison of gene expressions by $2^{-\Delta\Delta Ct}$ formula. The primer sequences of all PCR reactions were summarized in table 2.

Table 2 Summarized for primer sequences

Gene	Forward or reverse	5' → 3' sequence
MET	Forward primer	CTCCAgCATTTTTACggACC
	Reverse primer	gCTgCAAAgCTgTggTAAACT
TIMP4	Forward primer	ACgCCTTTTgACTCTTCCCT
	Reverse primer	ggCTCgATgTAgTTgCACAg
36B4	Forward primer	CTTCCCACTTgCTgAAAAg
	Reverse primer	CCAAATCCCATATCCTCgT

6. Transfection of green fluorescence protein *(GFP)* gene

KKU-M213 CCA cells were transfected with *GFP* gene by lentiviral infection system. This process was kindly performed by Dr. Bunpote Siridechadilok, Medical Molecular Biology Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, based on the previous protocol.[23] The transfection successful was checked under fluorescence microscope.

7. Animal

BALB/c-nu (immunocompromise) mice were cared in animal house of Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University. Cages were filtered-top cage (Figure 2) with wood shaving as bedding and changed 2-3 times per week. Food and water were given ad libitum and changed 2-3 times per week. The conditions were temperature = 25±2^{o}C, humidity = 60-70±10%, light = fluorescence standard 12:12 (day:night).

8. Cancer cell xenograft and estrogen treatment

Lot No. 1

GFP-transfected KKU-M213 CCA cells were subcutaneously injected into mice. Cell suspension was prepared as 1x10^{6} cells per 100 µl of phosphate buffer saline (PBS). The injection position was right paravertebral area. The amount of cell suspension that had been injected was 100 µl (1x10^{6} cells) each mouse. After injection, mice were divided into 3 groups. First group, 2 mice were fed with regular diet. Second group, 1 mouse was fed with regular diet plus 20 nmol 17β-estradiol (Sigma, E-4389) in aqueous solution per ball. Third group, 1 mouse was fed with regular diet plus 100 nmol 17β-estradiol in aqueous solution per ball. The estimation of feeding was 3 balls per mouse per day. So the second group, mouse would receive 60 nmol of 17β-estradiol per day and the third group would be 300 nmol per day.

Lot No. 2

GFP-transfected KKU-M213 CCA cells were subcutaneously injected into mice. Cell suspension was prepared as 4x10^{6} cells per 100 µl of phosphate buffer saline

(PBS). The injection position was right franking area. The amount of cell suspension that had been injected was 100 µl ($4x10^6$ cells) each mouse. After injection, mice were divided into 2 groups. First group, 2 mice were fed with regular diet. Second group, 2 mice were fed with regular diet plus 100 nmol 17β-estradiol in aqueous solution per ball. The estimation of feeding was 3 balls per mouse per day. So the second group, mice would receive 300 nmol of 17β-estradiol per day.

Figure 2 Top-filtered cages in this study

9. Analysis

After a period of housing, until tumor growth was significant, mice would be sacrificed by overdose of sodium pentobarbital intra-peritonial injection. Blood would be taken by cardiac puncture and serum would be sent for measuring of estradiol concentration by central lab of Siriraj hospital. Tumor would be measure its size and weight. Internal organs would be check for invasion and metastasis.

6. Results

Metastasis PCR array

The metastasis gene expression of KKU-M213 CCA cells treated or non-treated with estrogen were measured by metastasis PCR array using real time RT-PCR method. After compared between treated and non-treated cells, genes which down-regulated lower than 0.5 times or up-regulated higher than 2 times corresponded to the expected changes that could promote cancer metastasis would be included. The result showed that 13 genes of down-regulated and 11 genes of up-regulated group were included. The down-regulated genes which could be suppressed by estrogen were CDH11, CD82, BRMS1, TRPM1, RORB, CDH6, RB1, TNFSF10, TIMP4, CST7, MTSS1, COL4A2 and FAT. And the up-regulated genes which could be induced by estrogen were MMP10, ETV4, DENR, IL1B, RPSA, IL18, HRAS, SMAD2, MET, SYK and HPSE. Only TIMP4 and MET were selected for validation in next step while the other will be validated in the near future. The details of folding were shown in table 3.

Table 3 Ratio of metastasis gene expression in KKU-M213 CCA cells which treated or non-treated with 1 nM 17β-estradiol

	Gene	Expected change	E2 treated/ E2 non-treated ratio	Included
1	APC	down	1.806253	
2	BRMS1	down	0.000586	✓
3	CCL7	up	1.147107	
4	CD44	up	1.422077	
5	CDH1	down	1.662092	
6	CDH11	down	7.73E-06	✓
7	CDH6	down	0.076045	✓
8	CDKN2A	down	15.59487	
9	CHD4	down	2.018103	
10	COL4A2	down	0.317098	✓
11	CST7	down	0.239484	✓
12	CTBP1	down	0.612593	
13	CTNNA1	down	2.216065	
14	CTSK	up	0.138985	
15	CTSL1	up	0.350625	
16	CXCL12	up	0.614719	
17	CXCR4	up	0.067358	

	Gene	Expected change	E2 treated/ E2 non-treated ratio	Included
18	DENR	up	2.563296	✓
19	EPHB2	down	3.21095	
20	ETV4	up	2.493202	✓
21	EWSR1	up	0.133323	
22	FAT	down	0.394473	✓
23	FGFR4	up	0.081503	
24	FLT4	up	0.621144	
25	FN1	up	0.032872	
26	FXYD5	down	2.795294	
27	GNRH1	down	4.799886	
28	KISS1R	down	1.397647	
29	HGF	up	5.09E-07	
30	HPSE	up	142.8146	✓
31	HRAS	up	5.36285	✓
32	HTATIP2	down	5.767715	
33	IGF1	up	0.000545	
34	IL18	up	4.164086	✓
35	IL1B	up	3.324183	✓
36	IL8RB	up	0.645281	
37	ITGA7	down	195.7678	

	Gene	Expected change	E2 treated/ E2 non-treated ratio	Included
38	ITGB3	up	1.91587	
39	CD82	down	3.83E-05	✓
40	KISS1	down	4.310933	
41	KRAS	up	1.76296	
42	RPSA	up	3.429504	✓
43	MCAM	down	107.4856	
44	MDM2	up	1.383191	
45	MET	up	7.376825	✓
46	METAP2	up	0.000673	
47	MGAT5	up	0.008627	
48	MMP10	up	2.155466	✓
49	MMP11	up	1.545421	
50	MMP13	up	0.141905	
51	MMP2	up	1.147107	
52	MMP3	up	3.33E-06	
53	MMP7	up	0.000979	
54	MMP9	up	1.969733	
55	MTA1	up	0.906262	
56	MTSS1	down	0.25052	✓
57	MYC	up	1.295043	

	Gene	Expected change	E2 treated/ E2 non-treated ratio	Included
58	MYCL1	up	1.055554	
59	NF2	down	0.629815	
60	NME1	down	1.070289	
61	NME2	down	0.893785	
62	NME4	down	1.331452	
63	NR4A3	up	0.015072	
64	PLAUR	up	0.786217	
65	PNN	up	0.559806	
66	PTEN	down	0.656561	
67	RB1	down	0.132402	✓
68	RORB	down	0.004903	✓
69	SET	up	1.002082	
70	SMAD2	up	5.68831	✓
71	SMAD4	up	0.698823	
72	SRC	up	0.50278	
73	SSTR2	up	0.114467	
74	SYK	up	23.15096	✓
75	TCF20	up	0.589678	
76	TGFB1	up	0.647521	
77	TIMP2	down	0.738669	

	Gene	Expected change	E2 treated/ E2 non-treated ratio	Included
78	TIMP3	down	1.048262	
79	TIMP4	down	0.180241	✓
80	TNFSF10	down	0.160206	✓
81	TP53	up	0.723467	
82	TRPM1	down	0.002019	✓
83	TSHR	up	0.000106	
84	VEGFA	up	0.370617	

***In vitro* invasion assay**

KKU-M213 CCA cells which treated with 1 nM 17β-estradiol and 10 μM tamoxifen were checked their invasive property by *in vitro* invasion assay. The results showed that estrogen could promote the invasion of KKU-M213 CCA cells but the concentration of 17β-estradiol at 1 nM showed more invasive than at 10 nM. Moreover, tamoxifen could inhibit estrogen-induced invasion of KKU-M213 CCA cells. The results were summarized in table 4.

Table 4 Results from in vitro invasion assay of KKU-M213 CCA cells treated with estrogen and tamoxifen

CCA cells	17β-estradiol (nM)	Tamoxifen (μM)	Number of invading cells (Average ± SD of duplicated experiment)
KKU-M213	0	0	79 ± 43
	1	0	97 ± 23
	10	0	52 ± 8
	1	10	6 ± 1

Migration assay

KKU-M213 CCA cells which treated with 1 nM 17β-estradiol and 10 μM tamoxifen were checked their migratory property by wound healing migration assay. Photograph of artificial wound from each cell condition was taken and the distances of the scratches were measured. One example of the assay was shown in figure 3A. This experiment had been performed independently for 2 times and the results showed that at 6 hours estrogen could promote the migration of KKU-M213 CCA cells and this could be inhibited by tamoxifen. The results were shown in figure 3B.

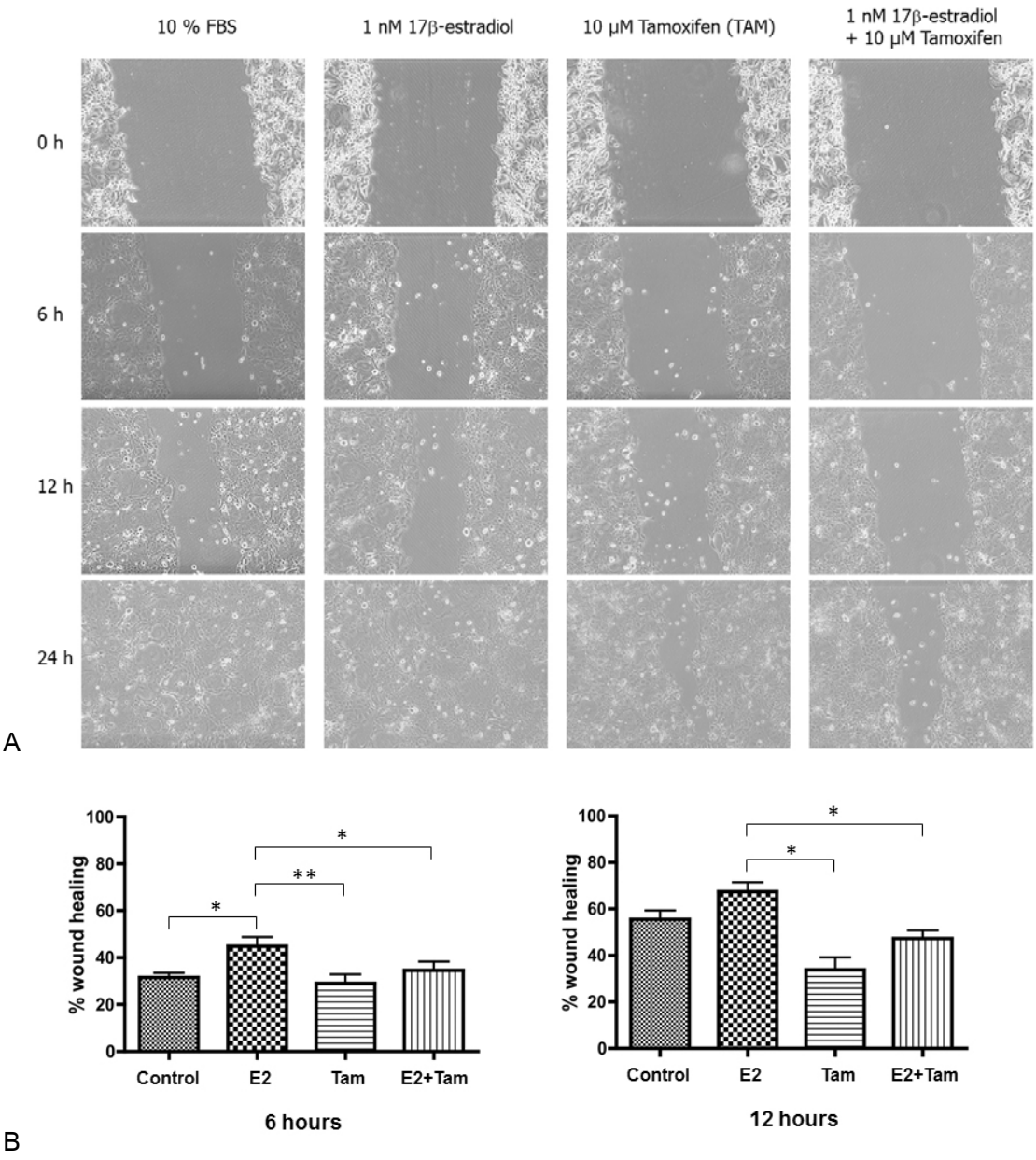


Figure 3 Wound healing migration assay of KKU-M213 CCA cells treated with 1 nM 17β-estradiol and 10 μM tamoxifen. A) Photograph from inverted microscope and B) The comparison of migration between conditions. * is the statistical significance at *P* value less than 0.05 and ** is the statistical significance at *P* value less than 0.001

***MET* and *TIMP4* gene expression in estrogen and tamoxifen treated CCA cells**

KKU-M213 CCA cells which treated with 1 nM 17β-estradiol and 10 μM tamoxifen and checked their migratory property by wound healing migration assay would be performed the RNA extraction and checked the expression of metastasis related gene *MET* and *TIMP4*. *MET* expression showed activated by estrogen and inhibited by tamoxifen, which correlated to the migration assay. In contrast *TIMP4* expression showed inhibited by estrogen and activated by tamoxifen, which inversed to the migration assay. The results were shown in figure 4.

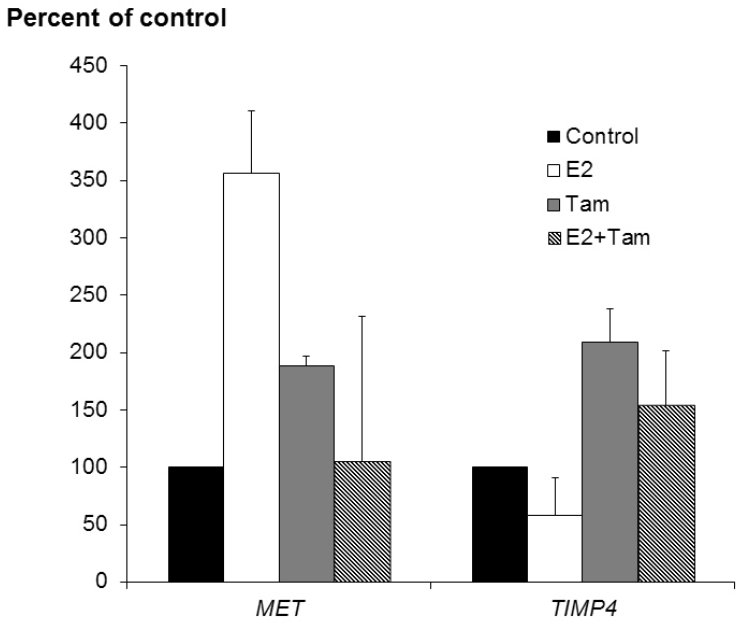
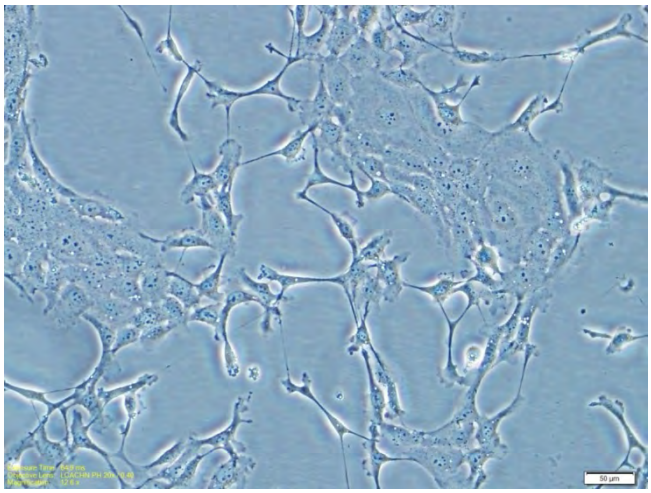


Figure 4 The expression of *MET* and *TIMP4* gene from KKU-M213 CCA cells treated with 1 nM 17β-estradiol and 10 μM tamoxifen after wound healing migration assay.

Transfection of *GFP* gene

KKU-M213 CCA cells were transfected with *GFP* gene by lentivirus system. The transfection was successful with transfection rate approximately 70% (Figure 5).

A



B

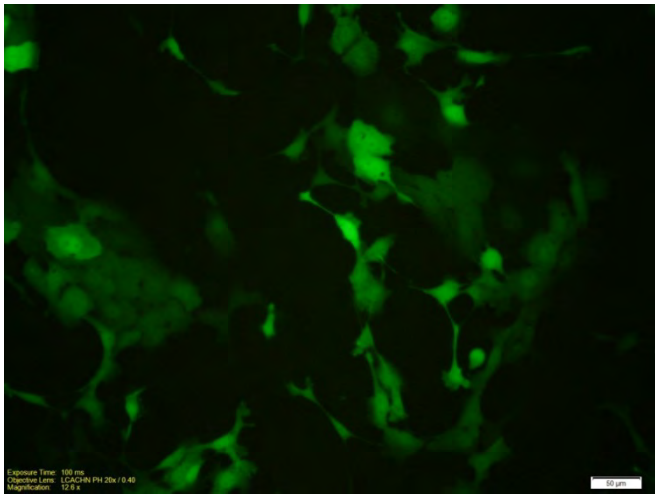


Figure 5 The expression of GFP in GFP-transfected KKU-M213 CCA cells. The original magnification was 200x. A) Photograph of cells under visible light. B) Photograph of green fluorescence (FITC channel). Exposure time was 100 ms.

BALB/c-nu xenograft with GFP-transfected KKU-M213 CCA cells

Lot No. 1

After injection of cancer cell in BALB/c-nu mice for 3 weeks, there was no evidence of tumor presented in each mouse. So cell injections were performed again with 100 µl of $4x10^6$ cells per 100 µl PBS. The injection sites were left franking region. In the 4^{th} week, the tumor presented on the right side of both first group-mice (1^{st} injection position) (Figure 6). The tumors grew quickly. At this time there was no tumor seen in 2^{nd} group- and 3^{rd}-group mice (Figure 6). The tumor of 3^{rd} group-mouse presented on the left side (2^{nd} injection position), after 4 days pass 2^{nd} injection. The tumor was markedly smaller than that of the 1^{st} group. There was no tumor presented in the 2^{nd} group-mouse. All mice were sacrificed in 5^{th} week (9 day after 2^{nd} injection) by overdose sodium pentobarbital intra-peritoneal injection. Bloods were withdrawn from cardiac puncture and the serum estradiol was measured (Table 5). Tumors were dissected and collected. Internal organs were screened the metastasis. Mice were photographed, however, under ultraviolet light tumors did not show green fluorescence (SYNGENE gel documentation G:BOX EF2) (Figure 7-10). In addition some tumor tissues were digested with trypsin, stained with propidium iodide (PI) and observed under fluorescence microscope, but there was no green fluorescence signal detected (Figure 11). There was no evidence of metastasis in lung and liver of each mouse. The tumors of 1^{st} group-mice were cystic structure with 0.5 ml serous content inside. The tumor of 3^{rd} group-mouse was solid structure. The data of all mice were summarized in Table 5. Tumor tissues from 3 mice were kept in RNAlater® solution and kept at $-20^{o}C$

for further genetic study. Tumor tissues from 1st group-mice were sent to make paraffin-embedded block and section with hematoxylin-eosin staining. The result showed as poorly differentiated adenocarcinoma with fibrosis in both mice (Figure 12 and 13). In tumor from mouse No. 1, the muscular invasion has been demonstrated. In tumor from mouse No. 2, the invasion of adrenal gland has been demonstrated. Tumor tissue from mouse No. 4 was less to be sent to pathological exam.

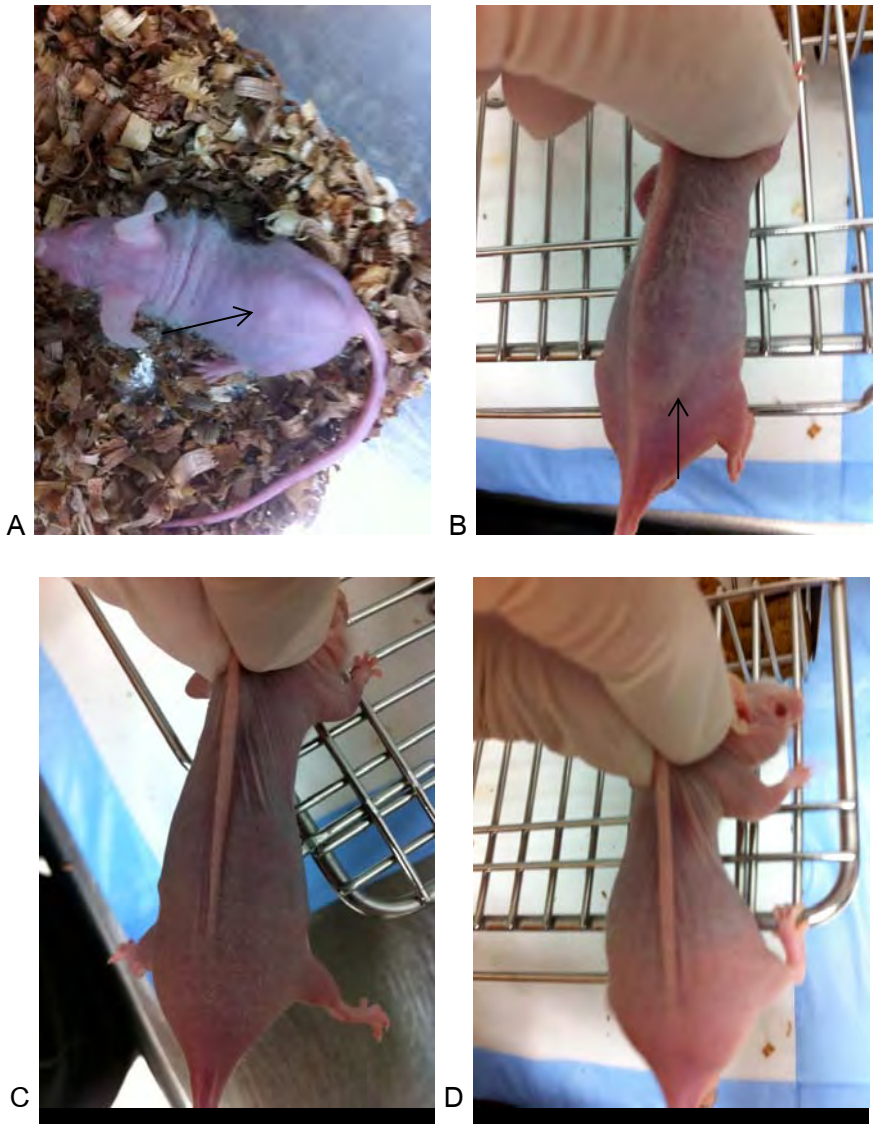


Figure 6 All mice at the time of 4th week after 1st injection. Large tumors were seen in 1st group-mice (arrow) (A and B) but not in 2nd group- (C) and 3rd group-mice (D).

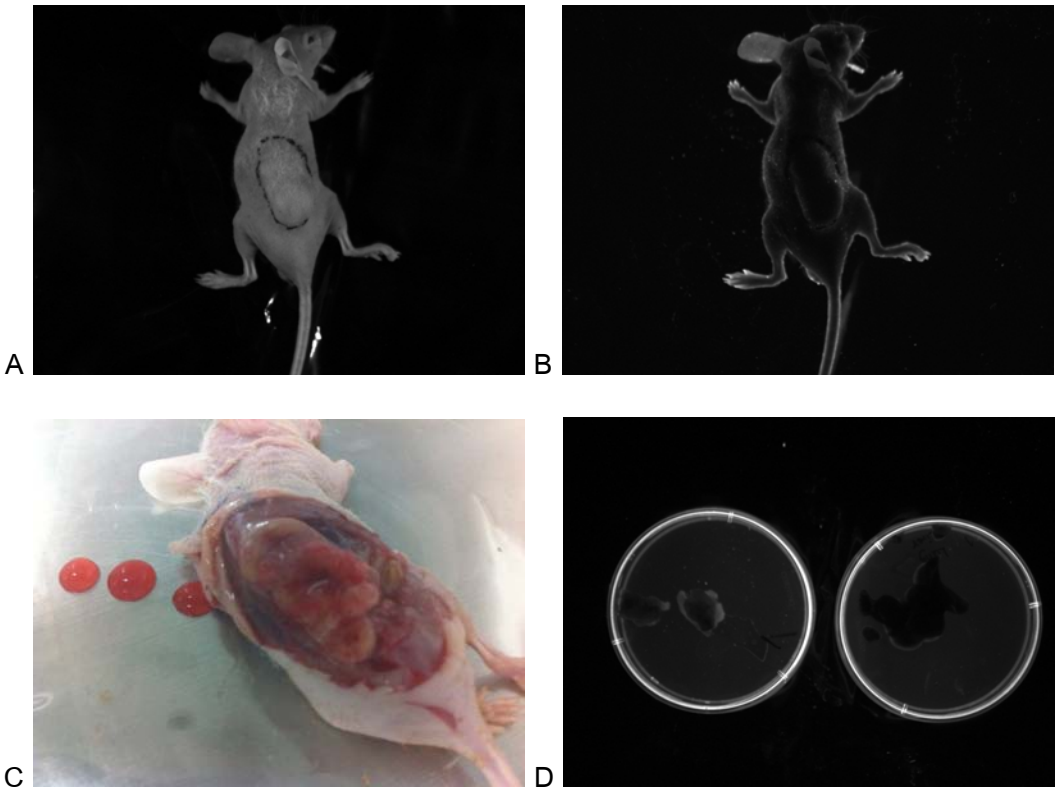


Figure 7 Mouse No. 1 after sacrified. A) Whole body with mark at tumor site. B) Photograph under UV light. There was no fluorescence signal from tumor site. C) Tumor with droplets of serous content beside. D) Tumor (left dish) and lung and liver (right dish). There was no fluorescence signal from tumor and internal organs.

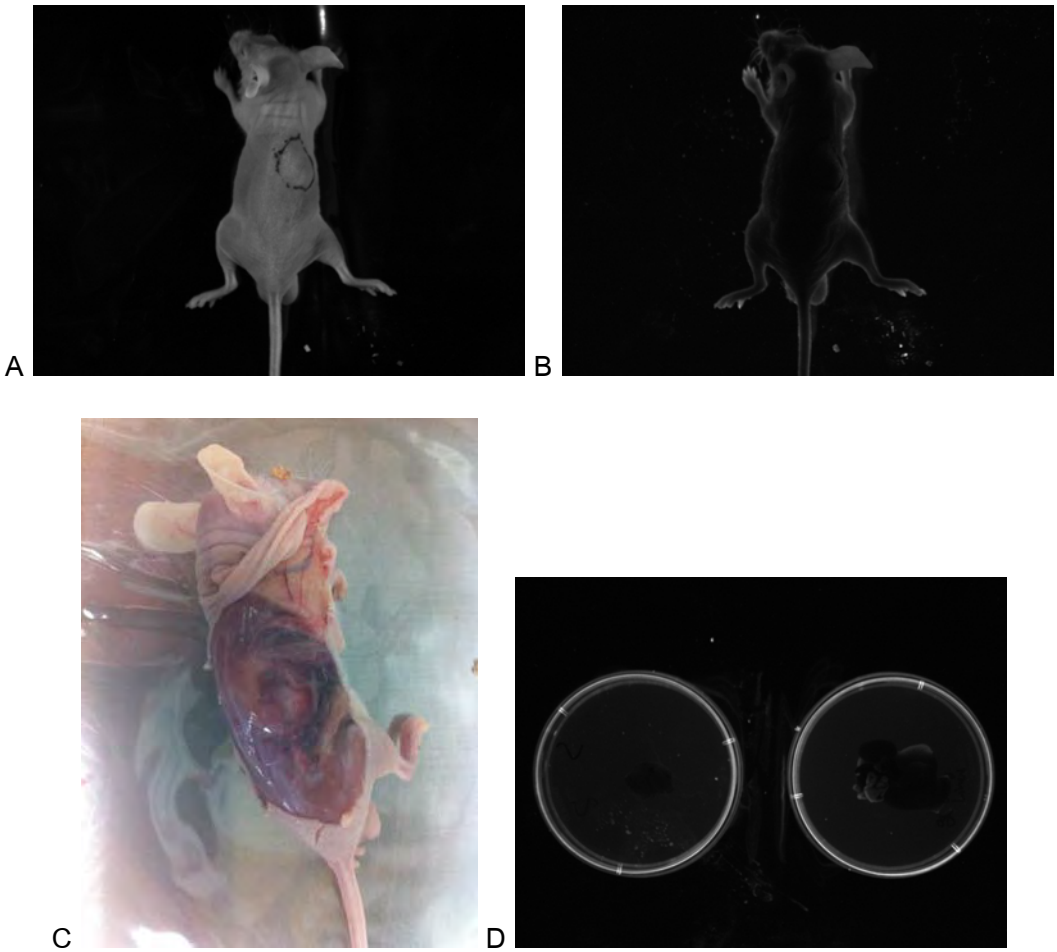


Figure 8 Mouse No. 2 after sacrified. A) Whole body with mark at tumor site. B) Photograph under UV light. There was no fluorescence signal from tumor site. C) Tumor. D) Tumor (left dish) and lung and liver (right dish). There was no fluorescence signal from tumor and internal organs.

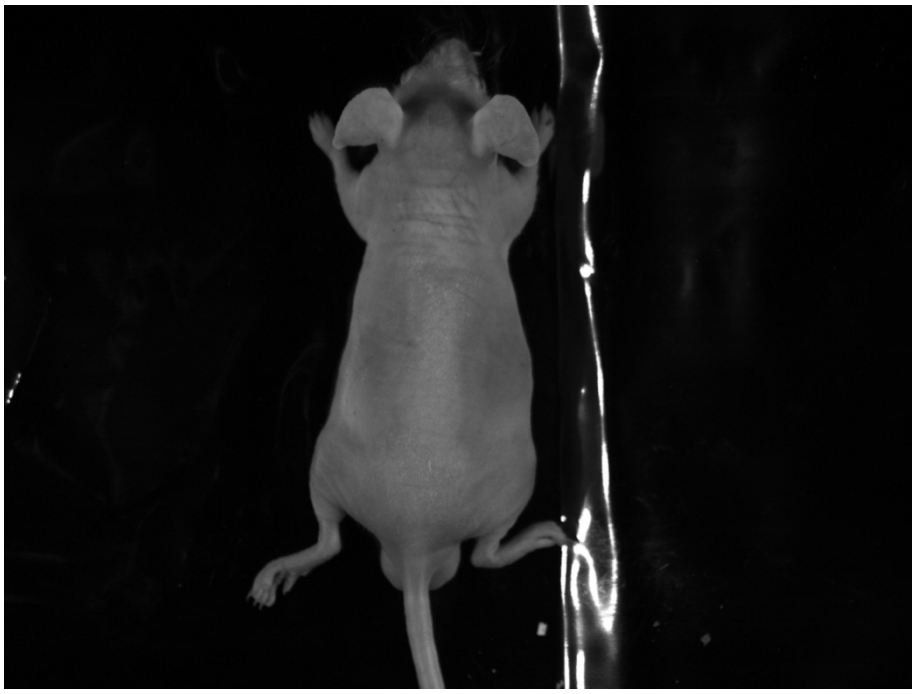
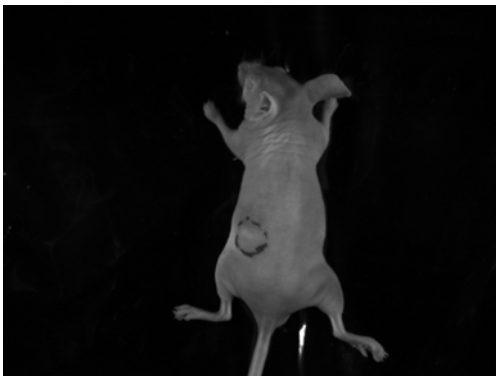


Figure 9 Whole body of mouse No. 3 after sacrified. There was no tumor seen.

A 

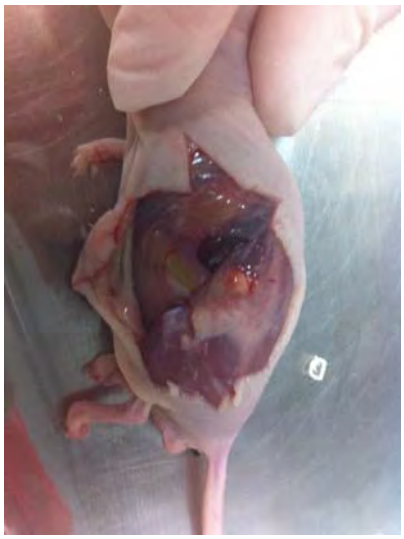
B 

Figure 10 Mouse No. 4 after sacrified. A) Whole body with mark at tumor site. B) Tumor presented on the left side.

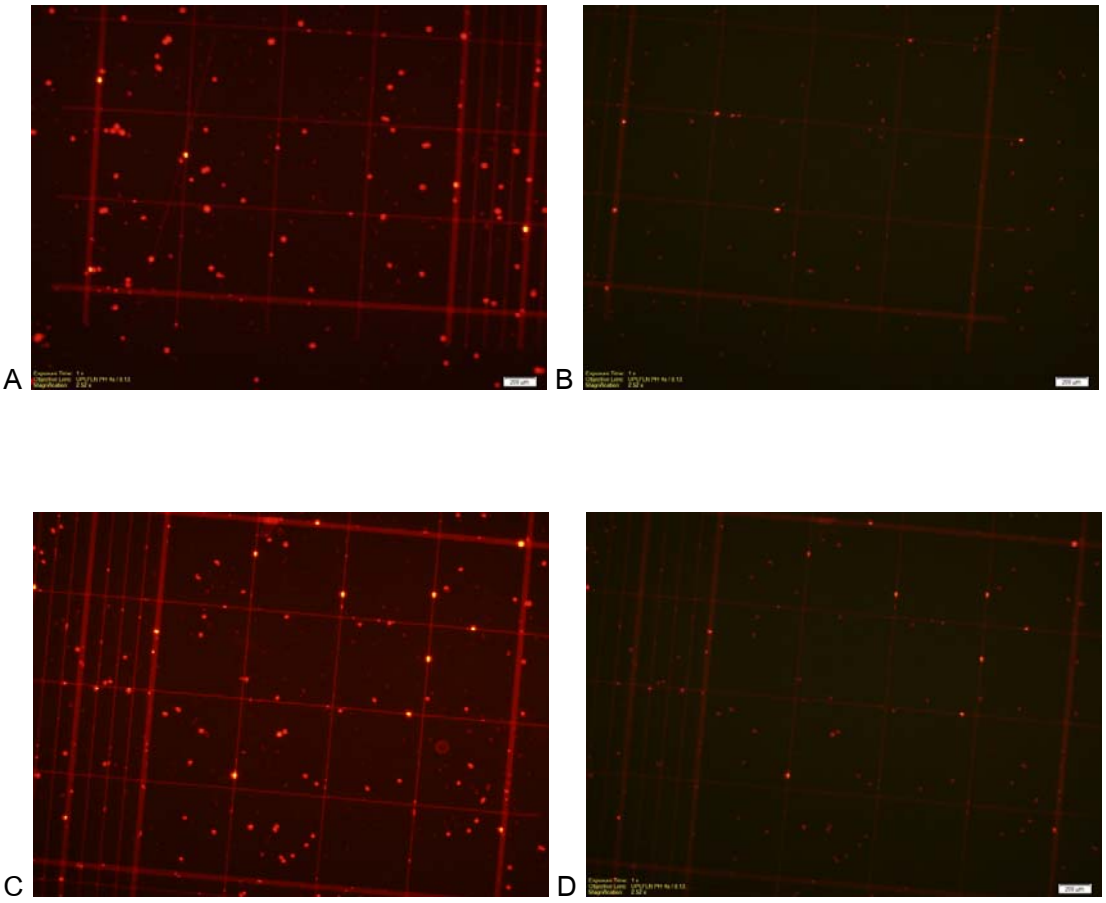
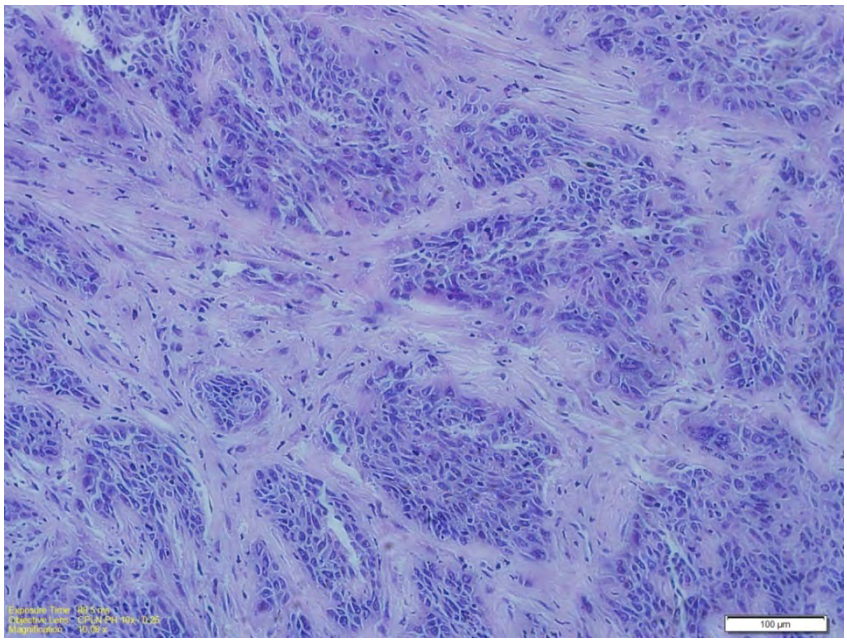


Figure 11 Fluorescence microscopic observations of cancer cells after digested from tumors of mouse No. 1 and 2 and stained with PI. A) Cells from mouse No. 1 in PI channel. B) Cells from mouse No. 1 in FITC channel. C) Cells from mouse No. 2 in PI channel. D) Cells from mouse No. 2 in FITC channel.

Table 5 Summary of mice data and tumor size from lot No. 1

Mouse No.	Group	Estrogen treatment (nmol/day)	Body weight (g)	Tumor size (cm x cm)	Tumor weight (g)	Serous content in tumor (ml)	Serum estrogen (pg/ml) / (nM)
1	1	0	21	2 x 3	1.52	0.5	127.45 / 0.468
2	1	0	22	2 x 3	1.34	0.5	150.40 / 0.552
3	2	60	26	-	-	-	141.75 / 0.520
4	3	300	25	0.8 x 0.8	0.84	-	274.65 / 1.008

A



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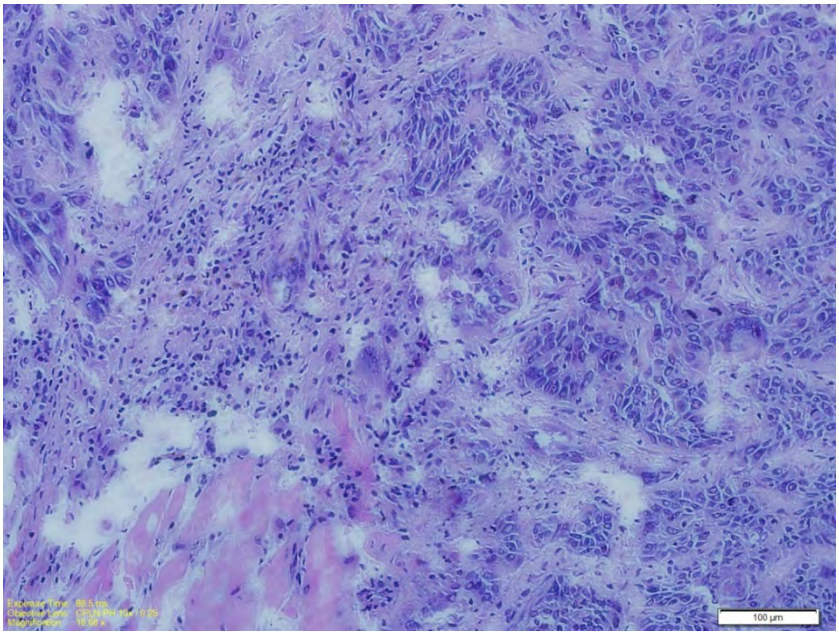
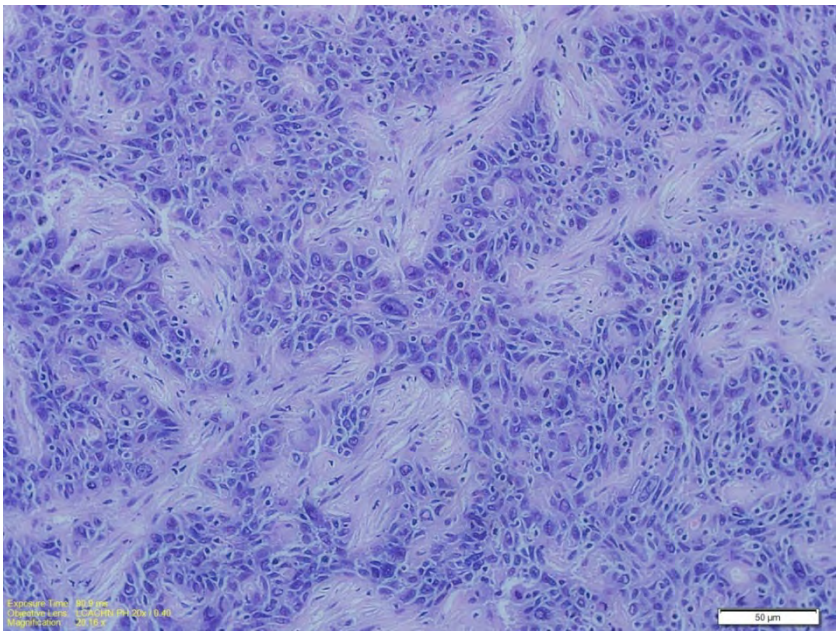


Figure 12 Histology of tumor tissue from mouse No. 1 with hematoxylin and eosin staining. The original magnification was 200x. A) Tissue structure showed poorly-differentiated adenocarcinoma morphology. B) Showed muscular invasion

A



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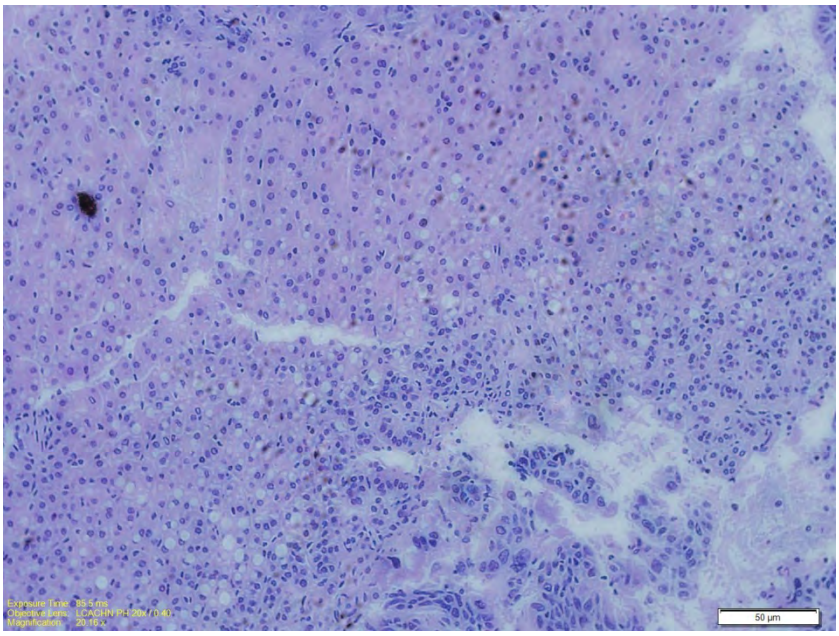


Figure 13 Histology of tumor tissue from mouse No. 2 with hematoxylin and eosin staining. The original magnification was 200x. A) Tissue structure showed poorly-differentiated adenocarcinoma morphology. B) Showed adrenal gland invasion

Lot No. 2

After injection of cancer cell in BALB/c-nu mice for 4 days, the tumor presented on the right side of one first group-mouse and both second group-mice. The tumors grew slower than that of lot No. 1 (of mice that presented as cystic tumor). At this time there was no tumor seen in the other mouse of first group (non-estrogen treated). By observation, the tumors in second group-mice were bigger than the tumor in first group-mouse. After 2 weeks from injection, both mice (with and without tumor) in the first group died then the experiment would be terminated. All mice in second group were sacrificed by overdose sodium pentobarbital intra-peritoneal injection. Bloods were withdrawn from cardiac puncture and the serum estradiol was measured (Table 6). Tumors were dissected and collected. Internal organs were screened the metastasis. Mice were photographed (Figure 14). In addition some tumor tissues were chopped and observed under fluorescence microscope and green fluorescence signals were detected (Figure 15). There were evidences of intraperitoneal metastasis in first group-mouse with primary tumor (Figure 16) and liver metastasis in first group-mouse without primary tumor (Figure 17). There was no evidence of metastasis in second group-mice. There was no presentation of cystic tumor in this lot. The data of all mice were summarized in Table 6. Tumor tissues were kept in RNAlater® solution and kept at -20°C for further genetic study. Internal organ (lungs, kidneys, liver and spleen) were collected and kept in -80°C. In addition tumor tissues were sent to make paraffin-embedded block.

Table 6 Summary of mice data and tumor size from lot No. 2

Mouse No.	Group	Estrogen treatment (nmol/day)	Body weight (g)	Tumor size (cm x cm)	Tumor weight (g)	Serum estrogen (pg/ml) / (nM)
1	1	0	22	0.9x1.7	0.26	NA
2	1	0	20	NA	NA	NA
3	2	300	23	1.5x1.7	0.32	94.3 / 0.346
4	2	300	27	1.0x1.0	0.80	136.1 / 0.5

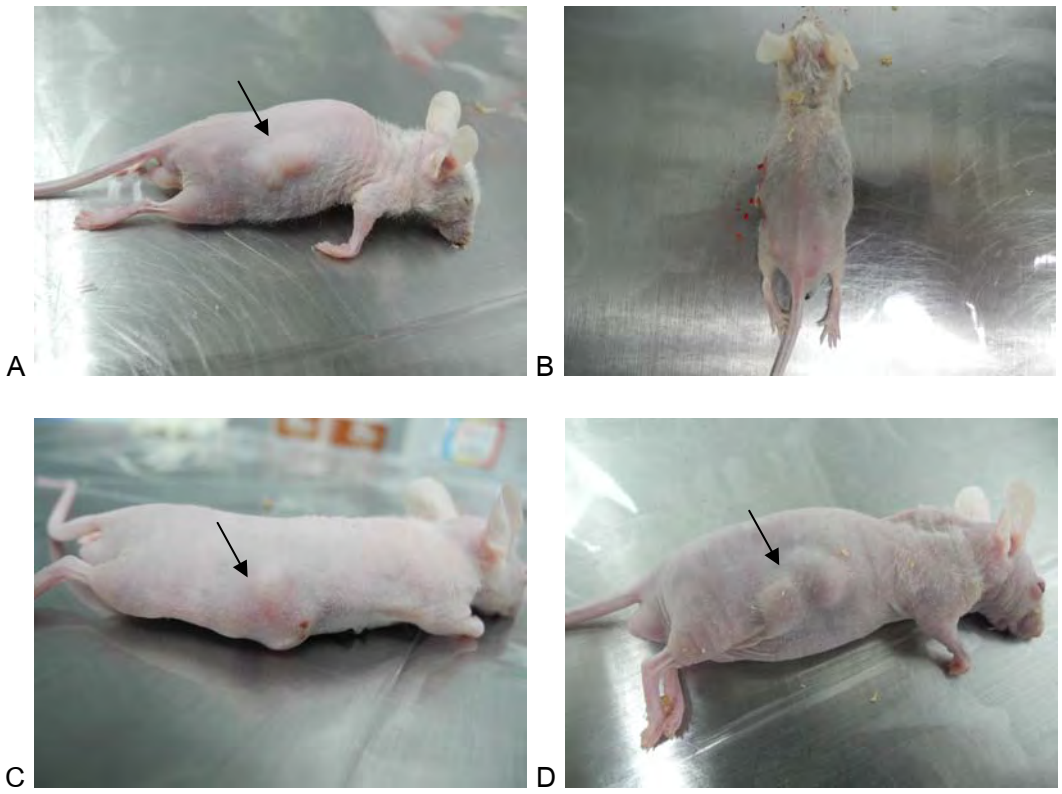
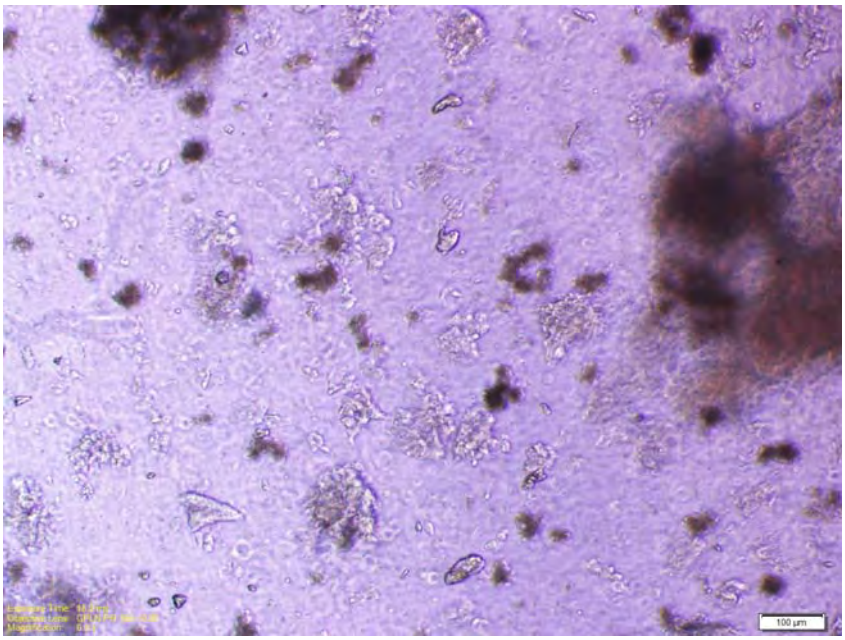


Figure 14 Tumor mass of all mice. A and B are mice from first group that no estrogen treatment applied A) Mouse No. 1 showed tumor mass at right franking region. B) Mouse No. 2 did not show tumor mass at right franking region. C and D are mice from second group that estrogen treatment applied C) Mouse No. 3 and D) mouse No. 4 showed bigger tumor mass at right franking region than mouse No.1.

A



B

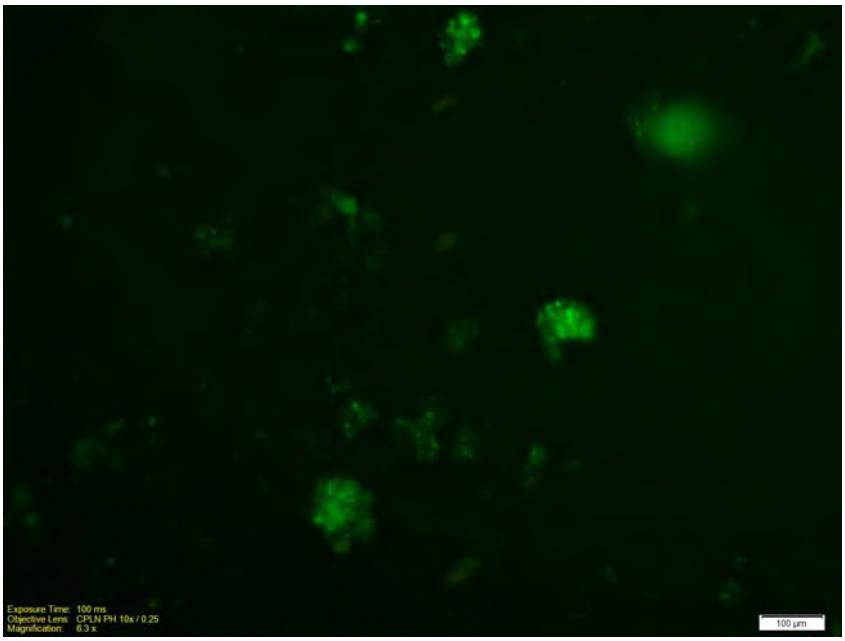


Figure 15 Green fluorescence signal from tumor mass. A) Bright field and B) FITC channel. The original magnification was 100x.

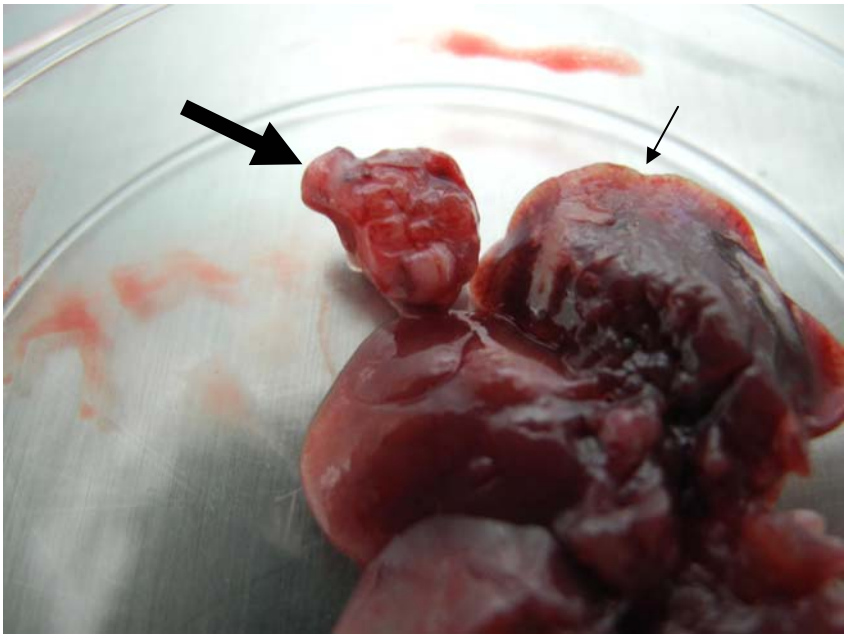


Figure 16 Metastasis tumor mass from intraperitoneal cavity (big arrow) of mouse No.1 and the liver edge that attached to this mass showed evidence of invasion (small arrow).

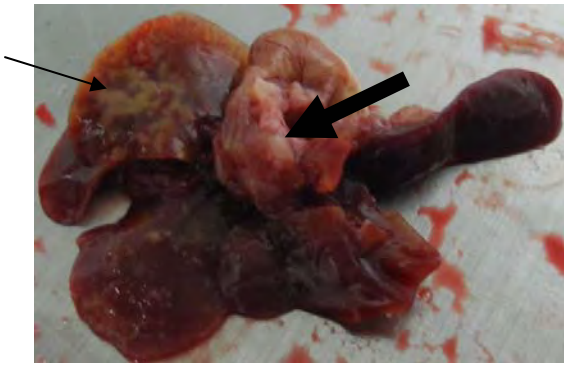


Figure 17 Metastasis tumor mass from liver hilar (big arrow) of mouse No.2 and the liver that showed evidence of invasion (small arrow).

7. Discussion

Estrogen is a steroid hormone in female reproductive system. In addition, in male metabolism, estrogen can be produced and function in male too. The main enzyme in estrogen synthesis is aromatase which is a member of cytochrome P450 system. The production of estrogen in male is mainly from adipose tissue which much lower than the production from ovary that occurs in female. The degradation and excretion of estrogen are mainly via biliary system so the biliary obstruction could produce the collection of estrogen in plasma. Our previous report showed that estrogen could be accumulated in CCA patients as associated with biliary obstruction status. Moreover the accumulation of estrogen in CCA patient showed association with short survival period. In addition estrogen showed biological effects on CCA cells *in vitro* including growth and invasion stimulation. To prove this phenomenon *in vivo*, animal experiment should be performed.

In this project, it was staring with the confirmation of estrogen effects on CCA cells invasion and screening of metastasis genes associated with estrogen stimulation. The result showed that by both *in vitro* invasion assay and wound healing migration assay showed that estrogen could induce this step of metastasis and tamoxifen could inhibit these processes. The screening of metastasis genes showed that there were some metastasis genes change more than 2 times when treated with estrogen. Some genes could promote metastasis and suspected to be increased after treatment and some could inhibit metastasis and expected to be decreased. The metastasis promoting genes which increased more than 2 times after treatment with estrogen were these 11

genes; MMP10, ETV4, DENR, IL1B, RPSA, IL18, HRAS, SMAD2, MET, SYK and HPSE. The metastasis suppression genes which decreased more than 2 times after treatment with estrogen were these 13 genes; CDH11, CD82, BRMS1, TRPM1, RORB, CDH6, RB1, TNFSF10, TIMP4, CST7, MTSS1, COL4A2 and FAT. Each gene is quite important but in this TIMP4 and MET were selected for validation, because both gene showed most consistency with estrogen treatment when performed real time RT-PCR (data not shown). The results from real time RT-PCR showed that estrogen could stimulate MET and inhibit TIMP4 expression and these processes could be disturbed by tamoxifen. This phenomenon was correlated to invasion or migration of KKU-M213 CCA cells in this experiment. Protein level is planned to be validated in animal model in the future.

The animal study in this project had been planned to be the immunosuppressive rat xenography but it changed to be nude mouse because the available of animal system and the difficult of the method. The BALB/c-nu is an appropriate animal for xenograph but the successful rate for implantation was also varying so the more animal is still needed. In this experiment, 4 mice have been used. GFP-labeled KKU-M213 CCA cells were injected into them. The labeling process was successful but the checking of cell characteristics change should be observed due to the using of lentivirus method could be leading to genetic disturbance. In lot No. 1, the injection of CCA cells in mice has been performed 2 times because that first time with $1x10^6$ cells of KKU-M213 CCA cells subcutaneous injection showed no effect for 4 weeks, than $4x10^6$ cells injection at the opposite site of the body should be performed. Two of them have been used as negative control and the others were treated with estrogen. The negative

control or the 1st group mice showed the tumor development at the first site of injection. This could be inferred that the tumor had slow progression at the first time and the second time injection was failure. It could be from the first injection site tumor could suppress the tumor growth of the other site or without estrogen at this time the tumor growth at the second injection site was not enough to see. The 2nd group mouse which has been fed with 20 nmol of 17β-estradiol/ball did not show the tumor development. This could be inferred that both injections were failure. The 3rd group mouse which has been fed with 100 nmol of 17β-estradiol/ball showed the tumor only at the second time injection site. It could be inferred that first time injection site was failure and the second time was success. It also could be referred that in short period the tumor could grow in the condition presence with estrogen faster than in the condition without or less estrogen. This experiment would not be concluded that estrogen could induce tumor formation or growth in animal model.

In lot No. 2 the tumor formation was successful in both group, estrogen treated and non-treated. In estrogen non-treated group there was only one mouse showed primary tumor at injected site but both of them showed metastasis in peritoneal cavity. Unfortunately serum estrogen level could not be measured in both mice of this group because the mice were dying before and the serum estrogen levels were unreliable. In mice with estrogen treated the tumor masses at primary site were bigger than the estrogen non-treated one but there was no evidence of metastasis in these mice. This could refer that estrogen has effect on tumor growth. In both mice of the estrogen non-treated group, the primary tumor were either small or absent, so it could be suggested that the secondary should has less effect from the control of primary site and showed as

metastasis. So it need to be confirmed this hypothesis and explored the mechanism. The results of experiment will be summarized and prepared for the manuscript.

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Output ที่ได้จากโครงการ

★★★★★

ประโยชน์ที่ได้รับ แบ่งเป็นประโยชน์ด้านต่างๆ ดังนี้

1. **ด้านวิชาการ** ผลของการวิจัยนี้จะได้ช่วยขยายขอบความรู้พื้นฐานเกี่ยวกับกลไกการแพร่กระจายของเซลล์มะเร็งท่อน้ำดี เป็นผลเบื้องต้นเพื่อที่จะขยายต่อเพื่อหาแนวทางการป้องกันการแพร่กระจายของเซลล์มะเร็งท่อน้ำดี โดยอาจหายาได้แก่ยาต้านเอสโตรเจน เช่น tamoxifen อาหารหรือชีวโมเลกุลจำเพาะ ที่ช่วยลดการแพร่กระจายของเซลล์มะเร็งก่อนที่จะลุกลามไปมาก เป็นการป้องกันแบบทุติยภูมิ (secondary chemoprevention) เพื่อให้การรักษาโดยการผ่าตัดได้ผลดี นอกจากนี้ ข้อมูลจากการวิจัยนี้จะเป็นพื้นฐานในการศึกษากลไกในการแพร่กระจายของมะเร็งท่อน้ำดีเพื่อใช้ในการควบคุมและรักษาโรค และยังสามารถประยุกต์เพื่อศึกษากลไกการแพร่กระจายของมะเร็งชนิดอื่นๆ อีกด้วย
2. **ด้านการแพทย์และสาธารณสุข** การวิจัยนี้จะช่วยให้เกิดการเปลี่ยนแปลงในแนวทางการรักษาโรคมะเร็งท่อน้ำดี อันเป็นโรคที่มีพยากรณ์โรคไม่ดี โดยที่แม้ว่าจะไม่สามารถป้องกันหรือลดความชุกของโรคได้ แต่ก็อาจจะช่วยให้ผู้ป่วยมีอายุยืนนานขึ้นและมีคุณภาพชีวิตที่ดีขึ้นได้ โดยการลดการแพร่กระจายของมะเร็งอันเป็นสาเหตุสำคัญที่ทำให้ผู้ป่วยเสียชีวิต
3. **ประโยชน์ต่อนักวิจัย** โครงการนี้เป็นโครงการวิจัยต่อเนื่องผู้วิจัย (นายปีติ ธุวจิตต์) ซึ่งทำให้สามารถเริ่มต้นงานวิจัยและพัฒนาตนเองเป็นนักวิจัยที่มีคุณภาพ และทำงานวิจัยในโครงการต่อๆ ไปได้ นอกจากนี้ยังทำให้ผู้วิจัยมีการติดต่อกับนักวิจัยที่มีคุณภาพทั้งจากในและนอกประเทศ อันจะส่งเสริมให้นักวิจัยสามารถทำงานวิจัยได้ดีต่อไป
4. **ประโยชน์ต่อบัณฑิตศึกษา** โครงการวิจัยนี้มีนักศึกษาระดับปริญญาเอก 1 คน คือ นายเอกพจน์ สิงห์สุขสวัสดิ์ หลักสูตรวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

5. **ประโยชน์ต่อสถาบันการศึกษาและวิจัย** โครงการวิจัยนี้ทำให้ได้ผลงานซึ่งได้นำเสนอในการประชุมระดับนานาชาติ (ดูภาคผนวก) และจะได้ตีพิมพ์ในวารสารระดับนานาชาติ ซึ่งนับได้เป็นผลงานของสถาบันการศึกษาและวิจัย ได้แก่ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น และ ศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดีมหาวิทยาลัยขอนแก่น รวมถึง granting agency ได้แก่ สำนักงานกองทุนสนับสนุนการวิจัย

ภาคผนวก

1. Manuscript

Under preparation

1. ชื่อ “The effects of 17β-estradiol on the proliferation, invasion and metastasis genes expression of cholangiocarcinoma cells *in vitro*” ได้จัดทำร่าง manuscript แล้วดังนี้

The effects of 17β-estradiol on the proliferation, invasion and metastasis genes expression of cholangiocarcinoma cells *in vitro*

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Abstract

The prevalence of cholangiocarcinoma, the carcinoma of bile duct epithelium, is low among worldwide, however, it is endemic in Thailand which associated with *Opisthorchis viverrini*, a liver fluke, infection. This type of cancer has poor prognosis with low 5-year survival rate due to its high metastasis rate which caused inadequate surgery while chemotherapy and radiotherapy are resisted. Cholangiocarcinoma is defined as a chronic liver disease with altered estrogen metabolism and could result in estrogen retention. Estrogenic response was known as a promoting factor in progression of some cancer, eg. breast cancer, including growth and invasion. In this study we tested the effects of 17β-estradiol, the most potent natural estrogenic substance, on proliferation and invasion of cholangiocarcinoma cell lines *in vitro*. Tamoxifen was used to be tested its inhibitory effect on 17β-estradiol stimulated cell invasion. The results showed the proliferative effect of 17β-estradiol on 4 cholangiocaricinoma cell lines (KKU-100, KKU-M055, KKU-M156 and KKU-M213) in dose dependent manner using the xCELLigence® cellular analysis system. The effect of 17β-estradiol on cholangiocarcinoma cell invasion showed highest at concentration 1 nM and this could be inhibited by tamoxifen. Metastasis genes expression of cholangiocarcinoma cell induced by 17β-estradiol was measured by RT^2 Prolifiler™ PCR array system. Total 84 metastasis genes expression were compared with normal control, 24 genes showed change in expression level more than twice which were 11 genes increased and 13 genes decreased in expression. The common pathway of estrogen induced metastasis genes in cholangiocarcinoma cells should be analyzed. The results should indicate the mechanism and control of

cholangiocarcinoma invasion and metastasis, which may be introduced to the new therapeutic guideline.

Keywords: Cholangiocarcinoma; 17β-estradiol; TFF1 trefoil protein; invasion

Introduction

Cholangiocarcinoma (CCA) is the carcinoma generated from bile duct epithelium [1]. Among worldwide CCA showed low prevalence, but it was raised each year [2]. Its etiology is chronic inflammatory conditions such as primary sclerosing cholangitis, hepatolithiasis, hepatitis C virus infection and liver fluke infection [3-6]. In Thailand CCA is an endemic carcinoma with caused serious public health problems especially in northeastern part and associated with a liver fluke *Opisthorchis viverrini* infection [6,7]. CCA showed high metastasis character which caused high mortality rate and bad prognosis because of its rapid metastasis and predisposes to a difficult or inoperable surgical outcome [8]. Median survival rate is low and the patients should die within a year of diagnosis [9]. Moreover it found frequently resisted to chemotherapy and radiation [10]. Previous study showed that CCA had impairment of estrogen metabolizing enzyme 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2) [11] that could lead to the accumulation of estrogen in plasma. Estrogen is a well-known biological substance which can stimulate tumor progression [12]. Estrogen itself could induce tumor progression include tumor growth and invasion [13,14]. Moreover estrogen has been reported as an agent that can induce expression of a set of metastasis genes [15].

CCA is a carcinoma which produce mucins and the major mucins produced are MUC1 and MUC5AC [16]. Expression of MUC1 is associated with shorter survival time [16]. MUC5AC was shown co-expressed with TFF1 trefoil protein, an estrogen responsive protein, in normal stomach mucosa [17]. TFF1 is a member of a trefoil factor family that includes in addition TFF2 and TFF3 and these three proteins are expressed in the gastrointestinal tract [18]. They play a role in protection against

damage of mucosa by stimulating epithelial cell migration and promote wound healing [18]. For TFF1 gene, it contains estrogen responsive element [19]. In normal tissues, TFF1 is highly expressed in the stomach and low expressed in breast, respiratory tract and tears [20-23]. TFF1 is not expressed in hepatocytes and cholangiocytes of small to medium size bile ducts and minimal expression was detected in large bile ducts in normal liver [24,25]. In pathological conditions, TFF1 has been reported its high level expression in inflammatory diseases, for examples, cholecystitis and inflammatory bowel disease [26,27]. TFF1 has been also reported its expression in many types of mucin-producing cancers, for examples, breast, ovarian, endometrium, prostate, thyroid, lung, stomach, pancreas, colon, gall bladder and CCA [28-38]. In cancer, TFF1 is suspected as a metastasis promoting agent in cancer due to its motogenic property [39]. There was a report of *in vitro* study showed that TFF1 can promote invasion of MCF-7 and MDA-MB231 human breast cancer cell lines [40]. Moreover TFF1 could stimulate KKU-100 and KKU-M213 CCA cell line in *in vitro* invasion assay with dose responsive manner [38]. In this study it showed both motogenic and chemoattractive pattern of CCA cell lines responded to TFF1 stimulation. These evidences support the possibility that estrogen could induce invasive property and metastasis of CCA in associate with TFF1 trefoil protein.

In this study we tested the effects of the most potent natural estrogenic substance, 17β-estradiol, on proliferation of 4 CCA cell lines, KKU-100, KKU-M055, KKU-M156 and KKU-M213 *in vitro*. We measured the invasive property of 2 CCA cell lines, KKU-100, and KKU-M213 using *in vitro* invasion assay. Tamoxifen was used to be tested its inhibitory effect on 17β-estradiol stimulated cell invasion. We also measured the expression of 84 metastasis genes in RT^2 Prolifiler^{TM} PCR array kit

in a CCA cell line, KKU-M213. The pathway analysis of estrogen-stimulated metastasis genes expression should be further performed.

Materials and Methods

CCA cell line. KKU100 CCA cell line was developed from poorly differentiated adenocarcinoma. KKU-M055 CCA cell line was developed from moderately differentiated adenocarcinoma. KKU-M156 CCA cell line was developed from moderately differentiated adenocarcinoma. And KKU-M213 CCA cell line was developed from well differentiated adenocarcinoma by Associate Professor Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University, THAILAND. Cells were cultured in phenol red-free DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum using penicillin and streptomycin for antibiotics and amphotericin B as an antifungal agent under the condition of 37^{o}C with 5% CO_2 and 90% humidity.

Proliferation assay. Real time proliferation assay was performed by xCELLigence® cellular analysis system (Roche, Mannheim, Germany). Briefly, CCA cells in phenol red-free DMEM without estrogen were seeded in 96-well plate with bottom electrode (Roche) as $1x10^3$ cells per 100 μl per well. After that phenol red-free DMEM with 17β estradiol at the concentrations of 0, 0.2, 2 or 20 nM in total volume of 100 μl were added. So the final concentrations of estrogen were 0, 0.1, 1 and 10 nM. The culture plate was installed in the xCELLigence® machine and incubate under the condition of 37^{o}C with 5% CO_2 and 90% humidity. Media were changed at 24- and 92-hour. The cell index was measured and plotted against time.

In vitro *invasion assay*. After change to phenol red- free medium for 48 hours, all cell lines were treated with 0, 1, 10 nM of 17β-estradiol with or without 0, 10 μM of tamoxifen for another 48 hours. The *in vitro* invasion assay was performed by BD Biocoated™-Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA).

The cells were prepared as 8 x 10^4 cells/ml cell suspension in phenol red-free DMEM with their conditions of 17β-estradiol. Approximately 4 x 10^4 cells were placed in the upper chamber above the Matrigel® layer, while the lower chambers were filled with the same media as upper chamber. The assays were performed in 5% CO_2 at 37°C for 28 hours. The cells that remained in the upper chamber and Matrigel® were removed and the cells that had invaded through the Matrigel® and attached to the lower part of the upper chamber were stained with crystal violet and counted under the light microscope. This experiment was performed duplicated.

RNA extraction and TFF1 expression assay. RNA extraction was performed by PerfectPure™ RNA Cell&Tissue kit (5 Prime, Hamburg, Germany). CDNA was produced using RT² First Strand Kits (SA Biosciences, Frederick, MD, USA). TFF1 mRNA expression was measured by real time RT-PCR using Light Cycler® 480 SyBR® green I Master (Roche) in Light Cycler® 480 machine (Roche) and 36B4 ribosomal protein gene was used as internal control. The forward and reverse primer sequences for TFF1 were CCCCTGGTGCTTCTATCCTAA and ATCCCTGCAGAAGTGTCTAAAA respectively. The forward and reverse primer sequences for 36B4 were CTTCCCACTTGCTGAAAAG and CCAAATCCCATATCCTCGT respectively.

Measurement of metastasis gene expressions. Metastasis genes expression of 17β-estradiol treated KKU-M213 CCA cell was measured by Human Tumor Metastasis RT^2 Prolifiler™ PCR array with RT² SYBR® Green qPCR Master Mixes (SA Biosciences). The procedure was performed following instruction manual from manufacturer. This array contained 84 genes assay which listed as following; APC (d), BRMS1 (d), CCL7 (u), CD44 (u), CD82 (d), CDH1 (d), CDH11 (d), CDH6 (d), CDKN2A (d), CHD4 (d), COL4A2 (d), CST7 (d), CTBP1 (d), CTNNA1 (d), CTSK

(u), CTSL1 (u), CXCL12 (u), CXCR4 (u), DENR (u), EPHB2 (d), ETV4 (u), EWSR1 (u), FAT (d), FGFR4 (u), FLT4 (u), FN1 (u), FXYD5 (d), GNRH1 (d), HGF (u), HPSE (u), HRAS (u), HTATIP2 (d), IGF1 (u), IL18 (u), IL1B (u), IL8RB (u), ITGA7 (d), ITGB3 (u), KISS1 (d), KISS1R (d), KRAS (u), MCAM (d), MDM2 (u), MET (u), METAP2 (u), MGAT5 (u), MMP10 (u), MMP11 (u), MMP13 (u), MMP2 (u), MMP3 (u), MMP7 (u), MMP9 (u), MTA1 (u), MTSS1 (d), MYC (u), MYCL1 (u), NF2 (d), NME1 (d), NME2 (d), NME4 (d), NR4A3 (u), PLAUR (u), PNN (u), PTEN (d), RB1 (d), RORB (d), RPSA (u), SET (u), SMAD2 (u), SMAD4 (u), SRC (u), SSTR2 (u), SYK (u), TCF20 (u), TGFB1 (u), TIMP2 (d), TIMP3 (d), TIMP4 (d), TNFSF10 (d), TP53 (u), TRPM1 (d), TSHR (u), VEGFA (u). The (d) determines the expectation that down-regulation of the gene could promote metastasis; while (u) determines the expectation that up-regulation of the gene could promote metastasis. Real-time PCR was performed using Light Cycler® 480 real time PCR machine (Roche). The gene expressions of KKU-M213 CCA cells treated and untreated with 17β-estradiol were compared and summarized.

Results

Real time proliferation assay

The effect of estrogen on CCA cell proliferation was determined by treating cells with various concentrations of 17β-estradiol and measured by xCELLigence® cellular analysis system. The concentrations of 17β-estradiol using in this experiment were 0, 0.1, 1 and 10 nM. The results showed that 17β-estradiol has proliferative induction effect on all 4 CCA cell lines, KKU-100, KKU-M055, KKU-M156 and KKU-M213 in dose dependent manner. KKU-M156 had minimally effect compared to other cell lines. The effect of 10 nM 17β-estradiol was higher than that of 1 nM in KKU-100 CCA cell, while in KKU-M213 it showed equal effect and in KKU-M055 it showed lower effect. Proliferative effect of 17β-estradiol on 4 CCA cell lines was showed in Fig. 1.

In vitro *invasion assay*

The *in vitro* invasion assay was performed with 2 CCA cell lines, KKU-100 and KKU-M213. Cells were treated with 0, 0.1, 1 and 10 nM of 17β-estradiol. The results showed that 17β-estradiol could stimulate invasion of both CCA cell lines in dose dependent manner. The maximum effect was showed at 1 nM concentration of 17β-estradiol and the effect was reduced at 10 nM similar in both cell lines. For KKU-100 CCA cell line, the cells treated with 1 nM 17β-estradiol showed 1.64 times more invasive than untreated cells and for KKU-M213 CCA cell line, the cells treated with 1 nM 17β-estradiol showed 1.64 times more invasive than untreated cells. Tamoxifen at 10 μM was used to test the inhibitory effect for invasion in both estrogen non-treated and treated cells. The results showed that tamoxifen could inhibit the invasion

of KKU-100 and KKU-M213 CCA cell lines in both estrogen non-treated and treated cells more than 50%. The invasion effect of 17β-estradiol on 2 CCA cell lines was showed in Fig. 2.

Measurement of TFF1 gene expressions

The expression of TFF1 gene was measured by real time RT-PCR and normalized with 36B4 gene expression. The results showed that KKU-100 and KKU-M213 CCA cell lines treated with 17β-estradiol and tamoxifen had changing in TFF1 expression as shown in Table 1. In both cell lines, the expression of TFF1 was increased in estrogen treated cells with dose dependent manner and the maximal expression was similar at 1 nM concentration. Tamoxifen could reduce the TFF1 expression in both cell lines.

Measurement of metastasis gene expressions

Metastasis genes expression of CCA cell induced by 17β-estradiol were measured by RT^2 Prolifiler^{TM} PCR array system. Total 84 metastasis genes expression were compared with normal control. The interesting genes should be have at least 2 times over- or under- expressed in 17β-estradiol treated control cells. The results showed that 24 genes showed change in expression level more than twice which were 11 genes increased and 13 genes decreased in expression, as showed in Table 1.

DISCUSSION

Estrogen is a multifunctional steroid which plays role in many type of cancer progression. It can promote tumor growth and metastasis. According to metastasis process, estrogen can induce a group of metastasis related genes including TFF1 trefoil protein. This protein has motogenic function and could promote tumor invasion [39]. In this study we examine the effect of estrogen on CCA cell invasion and the metastasis gene expression including TFF1 gene. The results indicate the tumor promoting effects of 17β-estradiol on CCA, including both proliferative and invasive induction. For proliferative induction, the responses were different among CCA cell lines. For invasive induction, the response of both CCA cell lines, KKU-100 and KKU-M213 showed similar with maximal at 1 nM 17β-estradiol. At 10 nM it showed reduced invasion that similar to the expression of TFF1 mRNA and the response to TFF1 *in vitro* as previous report [39]. Tamoxifen at 10 μM could inhibit the invasion of CCA cell lines.

For analysis of metastasis genes expression of KKU-M213 CCA cell line induced by 17β-estradiol compared with normal control, total 84 metastasis genes were analysed and 22 genes showed change in expression level as expected more than twice. There are 11 up-regulated genes. Heparanase (HPSE) has been reported the association with metastasis of melanoma and pancreatic cancer [41,42]. Spleen tyrosine kinase (SYK) has been shown inhibitory effect on breast cancer metastasis but promoted in squamous cell carcinoma of head and neck (oral cancer) [43,44]. Met proto-oncogene (MET) or hepatocyte growth factor/scatter factor receptor is the receptor for well-known metastasis promoting agent and met has been shown association with tumor metastasis and considered as target for cancer treatment [45].

V-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS) has been reported its association with aggressiveness of tumor including stomach cancer, hepatocellular carcinoma, breast cancer and brain tumor [46-49]. Interleukin 18 (IL18) is a pro-inflammatory cytokine which could promote anti-tumor immune system but itself could promote tumor progression including metastasis [50]. Ribosomal protein SA (RPSA) or 67-kDa laminin receptor has been reported the association with aggressiveness of CCA [51]. Density-regulated protein (DENR) in associated with Mct-1 oncoprotein can promote tumor progression [52]. Ets variant gene 4 (ETV4) has been reported the overexpression and promotion of aggressiveness of prostate cancer via production of matrix metallopeptidase 7 (MMP7) [53]. Matrix metallopeptidase 10 (MMP10) is the protease which can degrade extracellular matrix protein and promote metastasis. It has been reported in lung cancer and oral cancer [54,55].

For down-regulated genes, these gene expressions were usually suppressed during carcinogenesis process. So it could be less associated with tumor progression than up-regulated genes. However, it could be consider as indicators for identify regulatory pathway of estrogen stimulated metastasis in CCA. The 13 down-regulated genes were FAT tumor suppressor homolog 1 (FAT), collagen type IVα2 (COL4A2), metastasis suppressor 1 (MTSS1), cystatin F (CST7) or leukocystatin, TIMP metallopeptidase inhibitor 4 (TIMP4), tumor necrosis factor superfamily member 10 (TNFSF10), retinoblastoma 1 (RB1), cadherin 6 type 2 (CDH6) or K-cadherin, RAR-related orphan receptor B (RORB), transient receptor potential cation channel subfamily M member 1 (TRPM1), breast cancer metastasis suppressor 1 (BRMS1), CD82 molecule (CD82) and cadherin 11 type 2 (CDH11) or OB-cadherin. The analysis of estrogen stimulated metastasis in CCA including these down-regulated

genes should be further performed. Finally, the common pathway of estrogen induced metastasis genes in cholangiocarcinoma cells should be analyzed.

In conclusion, our work demonstrated the effect of estrogen on stimulation of proliferation in 4 CCA cell line, KKU-100, KKU-M055, KKU-M156 and KKU-M213. Estrogen can also promote TFF1 expression and invasion in KKU-100 and KKU-M213. The analysis of estrogen stimulated metastasis in KKU-M213 using metastasis RT^2 Prolifiler^{TM} PCR array system indicated 22 genes associated with invasive property induced by estrogen. The pathway of estrogen induced metastasis genes should be analyzed and the results should indicate the mechanism and control of cholangiocarcinoma metastasis for development of new therapeutic method.

Acknowledgements

This work was supported by the Research Strengthening Grant 2007 from National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) and a Mid-Career Grant (RMU5080055), Thailand Research Fund (TRF). The xCELLigence® cellular analysis system was supported by Roche Thailand Ltd. Parts of this project were done in Division of Molecular Biology in Medicine, Office of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok and Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

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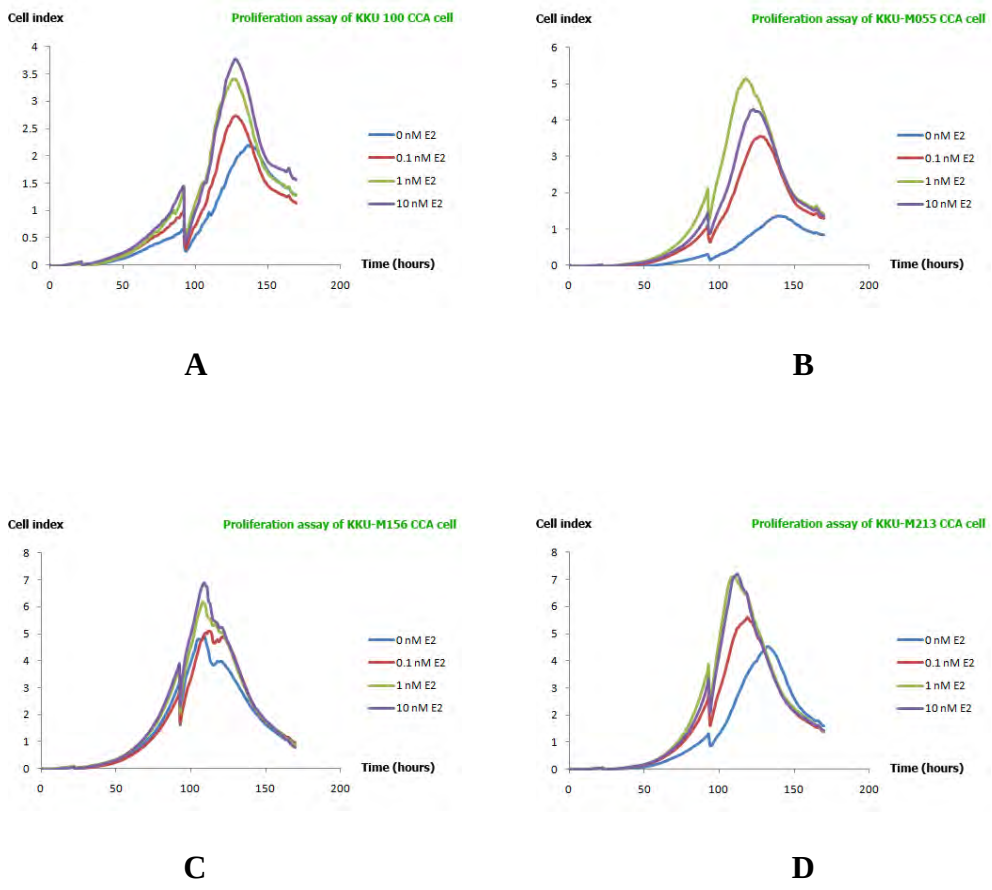


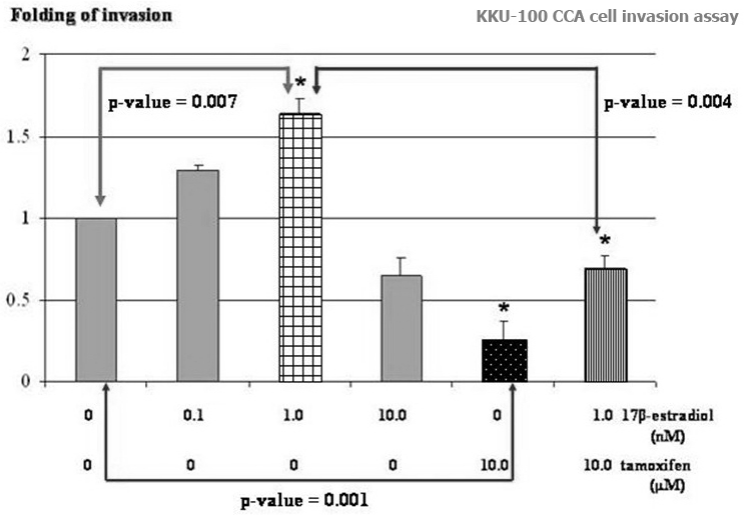
Fig. 1 Show proliferative effect of 17β-estradiol on 4 CCA cell lines. Y-axis showed cell signal index and X-axis showed time in hours. Media were changed at 24- and 92-hour, marked as dropped signal.

A = Proliferation assay of KKU-100 CCA cell line

B = Proliferation assay of KKU-M055 CCA cell line

C = Proliferation assay of KKU-M156 CCA cell line

D = Proliferation assay of KKU-M213 CCA cell line



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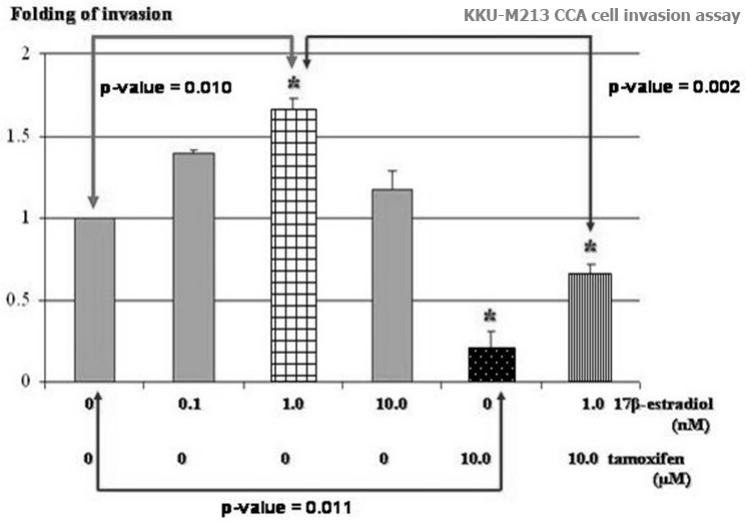

B

Fig. 2 Show effect of 17β-estradiol on invasive property of 2 CCA cell lines, KKU-100 and KKU-M213. Y-axis showed folding of invading cell of each condition compared to control (first column), X-axis showed condition of 17β-estradiol and tamoxifen concentrations used to treat cell.

A = *In vitro* invasion assay of KKU-100 CCA cell line

B = *In vitro* invasion assay of KKU-M213 CCA cell line

Table 2 The expression of TFF1 gene in KKU-100 and KKU-M213 CCA cell lines treated with various concentrations of 17β-estradiol and tamoxifen. The results showed folding of TFF1 gene compared to un-treated control.

Conditions	Folding of TFF1 expression	
	KKU-100	KKU-M213
0.1 nM 17β-estradiol	0.54	3.24
1.0 nM 17β-estradiol	2.34	4.55
10.0 nM 17β-estradiol	1.71	3.19
10.0 μM tamoxifen	0.72	0.39
1.0 nM 17β-estradiol + 10.0 μM tamoxifen	1.73	1.08
Un-treated control	1.00	1.00

Table 2 Metastasis gene change which greater than 2 fold induced by 17β-estradiol

Up-regulated genes			Down-regulated genes		
Symbol	**Name**	**Folding**	**Symbol**	**Name**	**Folding**
HPSE	Heparanase	142.8146	FAT	FAT tumor suppressor homolog 1	0.394473
SYK	Spleen tyrosine kinase	23.15096	COL4A2	Collagen, type IV, alpha 2	0.317098
MET	Met proto-oncogene (hepatocyte growth factor receptor)	7.376825	MTSS1	Metastasis suppressor 1	0.25052
SMAD2	SMAD family member 2	5.68831	CST7	Cystatin F (leukocystatin)	0.239484
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	5.36285	TIMP4	TIMP metallopeptidase inhibitor 4	0.180241
IL18	Interleukin 18 (interferon-gamma-inducing factor)	4.164086	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	0.160206
RPSA	Ribosomal protein	3.429504	RB1	Retinoblastoma 1	0.132402

	SA				
IL1B	Interleukin 1, beta	3.324183	CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)	0.076045
DENR	Density-regulated protein	2.563296	RORB	RAR-related orphan receptor B	0.004903
ETV4	Ets variant gene 4 (E1A enhancer binding protein, E1AF)	2.493202	TRPM1	Transient receptor potential cation channel, subfamily M, member 1	0.002019
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	2.155466	BRMS1	Breast cancer metastasis suppressor 1	0.000586
			CD82	CD82 molecule	3.83E-05
			CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	7.73E-06

2. ชื่อ “Mechanism and control of metastasis of cholangiocarcinoma cell stimulated by 17β-estradiol in animal model” กำลังรอผลการทดลองเพิ่มเติม

2. International presentation

1. Poster presentation

1.1 Peti Thuwajit, Chanitra Thuwajit. The effects of 17beta-estradiol on the proliferation, invasion and metastasis genes expression of cholangiocarcinoma cells *in vitro*. In the 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, October 15-17, 2009, Loutraki, Greece

Abstract

The prevalence of cholangiocarcinoma, the carcinoma of bile duct epithelium, is low among worldwide, however, it is endemic in Thailand which associated with *Opisthorchis viverrini*, a liver fluke, infection. In Thailand, the prevalence was about 20-time higher than in western country and may be more than 400-time in endemic area (northeastern part). This type of cancer has poor prognosis with low 5-year survival rate due to its high metastasis rate which caused inadequate surgery while chemotherapy and radiotherapy are resisted. Cholangiocarcinoma is defined as a chronic liver disease with altered estrogen metabolism and could result in estrogen retention. Estrogenic response was known as a promoting factor in progression of some cancer, eg. breast cancer, including growth and invasion. Our preliminary data showed that serum estrogen level increased in cholangiocarcinoma patients and associated with poor prognosis. In this study we tested the effects of 17β-estradiol, the most potent natural estrogenic substance, on proliferation and invasion of cholangiocarcinoma cell lines *in vitro*. Tamoxifen was used to be tested its inhibitory effect on 17β-estradiol stimulated cell invasion. The results showed the proliferative effect of 17β-estradiol on 4

cholangiocaricinoma cell lines (KKU-100, KKU-M055, KKU-M156 and KKU-M213) in dose dependent manner using the xCELLigence® cellular analysis system. The effect of 17β-estradiol on cholangiocarcinoma cell invasion showed highest at concentration 1 nM and this could be inhibited by tamoxifen. Metastasis genes expression of cholangiocarcinoma cell induced by 17β-estradiol was measured by RT^2 Prolifiler™ PCR array system. Total 84 metastasis genes expression were compared with normal control, 23 genes showed change in expression level more than twice which were 11 genes increased and 12 genes decreased in expression. The common pathway of estrogen induced metastasis genes in cholangiocarcinoma cells should be analyzed. The results should indicate the mechanism and control of cholangiocarcinoma invasion and metastasis, which may be introduced to the new therapeutic guideline.

1.2 Peti Thuwajit, Chanitra Thuwajit. Metastasis genes expression profile in cholangiocarcinoma cell induced by external estrogenic agent in associate with TFF1 trefoil protein. In the 5th International Conference on Tumor Microenvironment: Progression, Therapy & Prevention, During 20-24 October 2009, Versailles, France

Abstract

Cholangiocarcinoma is the carcinoma generated from bile duct epithelium. The prevalence of cholangiocarcinoma is low among worldwide, however it was raised each year. In Thailand cholangiocarcinoma is endemic especially in northeastern part and associated with a liver fluke *Opisthorchis viverrini* infection. The prognosis of

cholangiocarcinoma is quite poor because it has high metastasis rate. Previous study showed that cholangiocarcinoma had impairment of estrogen metabolizing enzyme that could leading to the accumulation of estrogen in plasma as we found in our preliminary study. Estrogen itself could induce tumor progression include tumor growth and invasion. TFF1 trefoil protein, an estrogen responsive protein, is a secreted protein that has motogenic effect and can promote cell migration and invasion. In this study we tested the effects of 17β-estradiol, the most potent natural estrogenic substance, on invasion and metastasis genes expression of cholangiocarcinoma cell lines *in vitro*. To test the role of TFF1 trefoil protein in estrogen-stimulated invasion, the permanent knockdown cholangiocarcinoma cell line and mock cell were generated and treated with 17β-estradiol. The results showed that 17β-estradiol could stimulate the invasion of cholangiocarcinoma cell but not in TFF1 knockdown cell compared to both negative control and mock control. Eighty-four tumor metastasis genes expression of estrogen treated cholangiocarcinoma cells (normal control, mock and TFF1 knockdown cell) was measured by RT^2 Prolifiler™ PCR array system. By compared between 3 cell groups, the result indicated 14 genes (CHD4, COL4A2, CST7, CTBP1, KISS1R, IL18, MET, MMP10, NF2, NME1, PTEN, TIMP2, TIMP4 and TRPM1) associated with invasive property induced by estrogen and TFF1 trefoil protein. The pathway of estrogen induced metastasis genes should be analyzed and the results should indicate the mechanism and control of cholangiocarcinoma metastasis for development of new therapeutic method.

1.3 Ekapot Singsuksawat, Chanitra Thuwajit, Peti Thuwajit. Tamoxifen inhibits migration of KKU-M213 cholangiocarcinoma cell line in association with metastasis genes expression. In The 96 Years of Opisthorchiasis: Past, Present and Future. International Congress of Liver Flukes. During 7 – 8 March 2011, Pullman Raja Orchid Hotel, Khon Kaen, Thailand

Abstract

Cholangiocarcinoma (CCA) is the cancer which associated with Opisthorchis viverrini, the liver fluke that endemics in Northeastern part of Thailand, infection. CCA has poor prognosis due to its rapid metastasis character, leading to inadequate surgery and systemic treatment. Metastasis is a molecular complex cascade which resulting in high mortal rate in cancer patients. The study in metastasis process becomes more and more remarkable in order to prevent or reduce the dissemination of cancer. From previous data, high estrogen level could be investigated in CCA patients, correlated to low survival time. It is leading to our hypothesis that estrogen could activate CCA progression and be inhibited by tamoxifen, the well-established estrogen antagonist using in clinical practice. To find a mechanism which regulates this complicated cascade in CCA cell line, the expression of metastasis-related genes would be identified. KKU-M213 CCA cell line was used by treated with 17β-estradiol and/or tamoxifen. Cell proliferation and migration were measured, whereas metastatic genes expression were investigated by realtime RT-PCR. The result showed that 17β-estradiol could stimulate KKU-M213 cell growth with dose-dependent manner while tamoxifen had limited effect on cell proliferation. The migration assay showed more increasing rate in KKU-M213 CCA cell line induced by 17β-estradiol, however this cannot be concluded because the

interference from proliferation effect could not be ruled out. On the other hand, tamoxifen could inhibit the migration without proliferating effect. Detection of gene expression has been performed tentative alteration in a group of metastasis-related genes; CHD4, COL4A2, CTBP1, IL-18, MET, MMP10, NF2, NME1, PTEN, TIMP2 and TIMP4. The results indicated that PTEN gene expression seem to be correlated with inhibiting KKU-M213 cell migration. Hence, the further investigation should be performed by manipulating this gene expression in which may reduce the metastasis in CCA cell and be beneficial in clinical practice.

1.4 Peti Thuwajit, Chanitra Thuwajit. The effects of 17β-estradiol on the proliferation, invasion and metastasis genes expression of cholangiocarcinoma cells in vitro. Mahidol-Kyoto Universities International Symposium 2010 “Integration of Biosciences for Innovative Medicine” During 16-17 December 2010, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract

The prevalence of cholangiocarcinoma, the carcinoma of bile duct epithelium, is low among worldwide, however, it is endemic in Thailand which associated with *Opisthorchis viverrini*, a liver fluke, infection. In Thailand, the prevalence was about 20-time higher than in western country and may be more than 400-time in endemic area (northeastern part). This type of cancer has poor prognosis with low 5-year survival rate due to its high metastasis rate which caused inadequate surgery while chemotherapy and radiotherapy are resisted. Cholangiocarcinoma is defined as a chronic liver disease with

altered estrogen metabolism and could result in estrogen retention. Estrogenic response was known as a promoting factor in progression of some cancer, eg. breast cancer, including growth and invasion. Our preliminary data showed that serum estrogen level increased in cholangiocarcinoma patients and associated with poor prognosis. In this study we tested the effects of 17β-estradiol, the most potent natural estrogenic substance, on proliferation and invasion of cholangiocarcinoma cell lines *in vitro*. Tamoxifen was used to be tested its inhibitory effect on 17β-estradiol stimulated cell invasion. The results showed the proliferative effect of 17β-estradiol on 4 cholangiocaricinoma cell lines (KKU-100, KKU-M055, KKU-M156 and KKU-M213) in dose dependent manner using the xCELLigence® cellular analysis system. The effect of 17β-estradiol on cholangiocarcinoma cell invasion showed highest at concentration 1 nM and this could be inhibited by tamoxifen. Metastasis genes expression of cholangiocarcinoma cell induced by 17β-estradiol was measured by RT^2 Prolifiler™ PCR array system. Total 84 metastasis genes expression were compared with normal control, 23 genes showed change in expression level more than twice which were 11 genes increased and 12 genes decreased in expression. The common pathway of estrogen induced metastasis genes in cholangiocarcinoma cells should be analyzed. The results should indicate the mechanism and control of cholangiocarcinoma invasion and metastasis, which may be introduced to the new therapeutic guideline.

1.5 Singsuksawat E, Thuwajit C, Thuwajit P. Estrogen could stimulate cholangiocarcinoma cell migration and be inhibited by tamoxifen. In the

Mahidol International Conference on Infections and Cancers 2012. During 6th-8th February 2012, The Landmark Hotel. Bangkok, Thailand

Abstract

Cholangiocarcinoma (CCA) is the cancer arising in bile duct. It has bad prognosis and difficult diagnosis owing to its character, rapid metastasis. This considerable attribute is a complex process which resulting in high mortality in cancer patients. Metastasis is still an unsolved problem which challenges to many researchers in order to prevent or reduce this dissemination. Unfortunately, the improvement of modalities in metastasis show yet limit efficacy. From earlier data, high estrogen level could be investigated in CCA patients, correlated to low survival rate. It is leading to our hypothesis that estrogen could stimulate CCA migration and be inhibited by tamoxifen, estrogen antagonist. Finding a critical metastasis-related gene that regulate this complicated cascade in CCA cell line would be identified. KKU-M213 and KKU-M156 CCA cell lines were used by treated with 17β-estradiol with or without tamoxifen. Cell migration was measured whereas metastatic gene expression was investigated by real time RT-PCR. The result showed that 17β-estradiol could stimulate KKU-M213 cell migration whereas tamoxifen showed inhibiting effect. However, this effect did not show significantly in KKU-M156 CCA cell. Detection of metastatic gene expression has been indicated that MET and TIMP4 gene alteration seem to be correlated with KKU-M213 cell migration but not KKU-M156. Hence, the further investigation should be performed by manipulating these gene expression in which may reduce the metastasis in CCA and be beneficial in clinical practice.

3. เอกสารรับรองจากคณะกรรมการจริยธรรมการวิจัยในสัตว์ทดลอง มหาวิทยาลัยมหิดล

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คณะกรรมการจริยธรรมการใช้สัตว์ทดลองเพื่องานทางวิทยาศาสตร์

เอกสารรับรองโครงการวิจัย

หมายเลข 011/2554

ชื่อโครงการภาษาไทย : การควบคุมการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีที่ถูกกระตุ้นโดยเอสโตรเจนในสัตว์ทดลอง

รหัสโครงการ : SI-ACUP 011/2554

หัวหน้าโครงการ / หน่วยงานที่สังกัด : ผู้ช่วยศาสตราจารย์ นพ. ปิติ ธุวจิตต์ / ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

สถานที่ทำวิจัย : คณะแพทยศาสตร์ศิริราชพยาบาล

เอกสารที่รับรอง :

1. โครงร่างการวิจัยตามใบสมัคร เลขที่ 011/2554
2. .....
3. .....

วันที่รับรอง : 28 กรกฎาคม 2554

วันที่หมดอายุ : 27 กรกฎาคม 2555

คณะกรรมการจริยธรรมการใช้สัตว์ทดลองเพื่องานทางวิทยาศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในสัตว์ที่เป็นสากล ได้แก่ International Guiding Principles for Biochemical Research Involving Animals.

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(ศาสตราจารย์ ดร.โกวิท พัฒนาปัญญาสัตย์)
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28 กรกฎาคม 2554
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วันที่

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(ศาสตราจารย์คลินิก นายแพทย์ธีรวัฒน์ กุลทนันทน์)
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Siriraj Animal Care and Use Committee (SI-ACUC)

Certificate of Approval

COA no. 011/2554

Protocol Title : Control of estrogen-stimulated cholangiocarcinoma cell metastasis in animal model

SI-ACUP number : SI-ACUP 011/2554

Principal Investigator / Affiliation : Asst. Prof. Dr. Peti Thuwajit/
Department of Immunology
Faculty of Medicine Siriraj Hospital, Mahidol University

Research site : Faculty of Medicine Siriraj Hospital

Approval includes :

1. ANIMAL CARE AND USE PROTOCOL No. 011/2554
2. .....
3. .....

Approval date : July 28, 2011

Expired date : July 27, 2012

This is to certify that Siriraj Animal Care and Use Committee is in full Compliance with International Guidelines for Animal Research Protection such as International Guiding Principles for Biochemical Research Involving Animals.

K. Pattanapanyasat
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(Prof. Dr.Kovit Pattanapanyasat)
Chairman

July 28, 2011
.....
date

T. Kulthan
.....
(Clin. Prof.Teerawat Kulthanan, M.D)
Dean of Faculty of Medicine Siriraj Hospital

02 ส.ค. 2554
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date