KRAS mutations in endometrial endometrioid adenocarcinoma

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Abstract

Endometrial carcinoma is the third most common gynecologic malignancy in Thailand. The prevalence of endometrial cancer seems to be increasing in younger Thai patients with mean age of 40 years and have risk factor of obesity. KRAS oncogene is one of the genetic alterations and plays an important role in the mechanism of carcinogenesis in endometrial cancer, especially endometrioid type. KRAS mutations are found in 10% to 30% of endometrial carcinomas; however, the relationship between KRAS mutations and clinicopathological variables remains unclear. The aims of this study were to examine the prevalence of KRAS mutations in Thai patients with endometrial endometrioid adenocarcinoma, to investigate the correlation between the mutations and clinicopathogical variables, and to screen KRAS mutations using reverse dot blot hybridization technique. A total of 190 patients with endometrioid adenocarcinoma were analyzed for KRAS exon 2 mutation using direct sequencing. Thirty selected samples were tested using reverse dot blot hybridization assay. Statistical correlation of KRAS mutation with clinicopathogical variables was evaluated. KRAS mutations were detected in 37 of 190 cases (19.5%). Thirty three (17.4%) of 190 cases were missense mutation, whereas 4 cases (2.1%) showed silent change. The frequent mutations were detected in codon 12 and 13. There were 9 cases of missense mutations detected at other sites of exon2, including codon 3, 14, 15, 27, 29, 33, and 34. Trend of KRAS mutations was found in patients with grade1-2 and stage I-II, but none of significant correlation. There was no significant relationship between the presence of KRAS mutations and clinicopathological variables (age, BMI, parity, depth of myometrial invasion, LVSI, uterine cervical and lymph node involvement, and synchronous ovarian cancer/ovarian metastasis). Detecting all of KRAS mutations in 30 selected cases, both direct sequencing and reverse dot blot hybridization assay were found to have similar results. In conclusion, this study suggests that the presence of KRAS mutations may be a relatively common event in endometrial carcinogenesis in subgroup of Thai patients, but their prognostic value is limited. With an alternative tool applicability comparing to direct sequencing, reverse dot blot hybridization is an effective and reliable assay for screening of KRAS point mutations in FFPE tissues.

การศึกษาการกลายพันธุ์ของยีน KRAS ในมะเร็งเยื่อบุโพรงมดลูกชนิดเอนโดแมทริออยด์

บทคัดย่อ

มะเร็งเยื่อบุโพรงมดลูกพบมากเป็นอันดับสามของมะเร็งในระบบอวัยวะสืบพันธุ์สตรีในประเทศไทย ความชุกของโรคมะเร็งเยื่อบุโพรงมดลูกมีแนวโน้มจะเพิ่มสูงขึ้นในผู้ป่วยไทยที่มีอายุน้อยเฉลี่ยกว่า 40 ปี และมีปัจจัยเสี่ยงของโรคอ้วน การศึกษาระดับอณูชีวโมเลกุลพบว่ายีน KRAS มีความสัมพันธ์กับกลไกของการเกิดมะเร็งเยื่อบุโพรงมดลูกโดยเฉพาะชนิดเอนโดแมทริออยด์ มีรายงานการศึกษาพบ KRAS mutation ร้อยละ 10 ถึง 30 ในมะเร็งเยื่อบุโพรงมดลูก อย่างไรก็ตาม การศึกษาความสัมพันธ์ของการเกิด KRAS mutation กับปัจจัยทางคลินิกและพยาธิวิทยายังไม่ชัดเจน การวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อหาความชุกของการเกิด KRAS mutation ในมะเร็งเยื่อบุโพรงมดลูกชนิดเอนโดแมทริออยด์ในผู้ป่วยชาวไทย และศึกษาความสัมพันธ์ของการเกิด KRAS mutation กับปัจจัยต่าง ๆ ทางคลินิกและพยาธิวิทยา รวมถึงการตรวจคัดกรอง KRAS mutation ด้วยเทคนิค reverse dot blot hybridization ตัวอย่างการศึกษาเป็นกลุ่มผู้ป่วยมะเร็งเยื่อบุโพรงมดลูกชนิดเอนโดแมทริออยด์จำนวน 190 ราย ทำการวิเคราะห์หา KRAS mutation บริเวณ exon 2 ด้วยวิธี direct sequencing และวิเคราะห์หาความสัมพันธ์ทางสถิติของการเกิด KRAS mutation กับปัจจัย ทางคลินิกและพยาธิวิทยา และทำการศึกษาตัวอย่างจำนวน 30 ราย โดยนำมาตรวจสอบการเกิด KRAS mutation ด้วยเทคนิค reverse dot blot hybridization ผลการศึกษาตรวจพบ KRAS mutation ในผู้ป่วย 37 ราย จาก 190 ราย คิดเป็นร้อยละ 19.5 โดย 33 ราย เกิดแบบ missense mutation และอีก 4 รายเกิดแบบ silent change ตำแหน่งของการเกิด mutation ที่พบได้บ่อยคือตำแหน่ง codon 12 และ codon 13 นอกจากนี้ยังตรวจพบการเกิด missense mutation ตรงตำแหน่งอื่นๆ ของบริเวณ exon 2 ได้แก่ codon 3, 14, 15, 27, 29, 33 และ codon 34 การเกิด KRAS mutation มีแนวโน้มบพได้บ่อยในผู้ป่วย grade 1-2 และ stage I-II แต่ไม่มีความสัมพันธ์อย่างมีนัยสำคัญ ปัจจัยอื่นๆ ทางคลินิกและพยาธิวิทยาได้แก่ อายุ ค่า BMI, การมีบุตร depth of myometrial invasion, LVSI, uterine cervical and lymph node involvement, and synchronous ovarian cancer/ovarian metastasis พบไม่มีความสัมพันธ์กับ KRAS mutation อย่างมีนัยสำคัญ การศึกษาตัวอย่างจำนวน 30 ราย ด้วยวิธี reverse dot blot hybridization ให้ผลสอดคล้องทุกรายกับการตรวจด้วยวิธี direct sequencing สรุปการศึกษานี้แสดงให้เห็นว่าการเกิด KRAS mutation น่าจะมีความเกี่ยวข้องกับกระบวนการเกิดมะเร็งเยื่อบุโพรงมดลูกในกลุ่มผู้ป่วยไทยจำนวนหนึ่ง แต่ไม่มีผลต่อการพยากรณ์โรค สำหรับวิธีทางเลือกที่นำมาประยุกต์ใช้ในการตรวจ KRAS mutation เมื่อเปรียบเทียบกับวิธี direct sequencing การตรวจด้วยวิธี reverse dot blot hybridization

ถือเป็นวิธีที่มีประสิทธิภาพและเชื่อถือได้ในการตรวจคัดกรอง KRAS mutation ในชิ้นเนื้อมะเร็งที่เป็นชนิดฝังพาราฟิน (formalin-fixed, paraffin-embedded tissue)

Introduction

Endometrial carcinoma is the third most common gynecologic malignancy in Thailand with the incidence rate of 4/100,000/years (Khunhaprema et al., 2013). Most of patients are menopause with the average age of diagnosis around 60 years. It has been reported that obesity was only the independent factor associated with endometrial cancer in Thai patients aged 45 years or younger (Hanprasetpong et al., 2008 and Manchana and Khemapech, 2008). The major type of endometrial carcinomas is endometrioid adenocarcinoma. The endometrioid type not only relates to endogenous or exogenous hyperestrogenic stimulation, but also relates to obesity, diabetes, hypertension, nulliparity, and chronic anovulation (Dahlgren et al., 1991; Doll et al., 2008). The carcinogenesis of endometrioid type involves a wide variety of molecular alterations, consisting of microsatellite instability, PTEN mutation, KRAS mutation, and β-catenin mutation (Bansal et al., 2009).

Alterations of KRAS gene are considered to be one of many molecular factors detected in several human cancers, including endometrial cancer. The RAS family of oncogenes is constituted of three members, known as KRAS, HRAS, and NRAS. The KRAS oncogene encodes a 21 kDa guanine nucleotide-binding protein (G-protein) that has a central role in the regulation of cell proliferation and differentiation (Friday and Adjei, 2005). Mutations of KRAS gene result in constitutive signal transduction pathway and subsequently effect on upregulated cell proliferation, cell death, and cancer progression (Kiaris and Spandidos, 1995). Previous studies have shown that KRAS gene mutations were identified in 10% to 30% of endometrial carcinomas (Sasaki et al., 1993; Duggan et al., 1994; Lax et al., 2000; Dobrzycka et al., 2009). The point mutations were predominantly found in codons 12 and 13 (Mammas et al., 2005). KRAS mutation has been documented that it associates with submicroscopic myometrial invasion and can be used to assess the depth myometrial invasion in stage I endometrial cancer (Alexander-Sefre et al., 2003). Furthermore, some studies have identified the mutations in endometrial hyperplasia, suggesting that KRAS mutation may be an early event in the pathogenesis of endometrium (Sasaki et al. 1993; Dobrzycka et al., 2009).

Nowadays, there are several methods to detect KRAS point mutations. Although DNA sequencing remains the gold standard for detecting the KRAS mutation, DNA extracted from FFPE tissues is poor quality and it is short DNA fragmentations for use as DNA template. Thus, reverse dot blot hybridization assay is an alternative method to be applied in detection of KRAS mutation in FFPE samples.

However, the detection of KRAS mutations in endometrial carcinoma in Thai patients have not yet been investigated. Therefore, the aims of this study were to examine the prevalence of KRAS mutations in Thai patients with endometrial endometrioid adenocarcinoma, to investigate the correlation between the mutations and clinicopathogical variables, and to screen KRAS mutations using reverse dot blot hybridization technique.

Statement of Aims

The specific aims of this study were:

Aim 1. To examine the prevalence of KRAS mutations in Thai patients with endometrial endometrioid adenocarcinoma.

Aim 2. To investigate the correlation between the mutations and clinicopathogical variables.

Aim 3. To screen KRAS mutations using reverse dot blot hybridization technique.

Review of Literature

Endometrial carcinoma is the third most common gynecologic malignancy in Thai population after cervical cancer and ovarian cancer with relative frequency of 3.6% (Attasara et al., 2011). The estimated incidence rate of endometrial carcinoma is 4.0 per 100,000 female populations (Khunhaprema et al., 2013). Most of patients are menopause with the average age of diagnosis around 60 years. The major type of endometrial carcinomas is endometrioid adenocarcinoma (Amant et al., 2005). The endometrioid type not only relates to endogenous or exogenous hyperestrogenic stimulation, but also relates to obesity, diabetes, hypertension, nulliparity, and chronic anovulation (Dahlgren et al., 1991; Doll et al., 2008). The carcinogenesis of endometrioid type involves a wide variety of molecular alterations, consisting of microsatellite instability, PTEN mutation, KRAS mutation, and β-catenin mutation (Bansal et al., 2009).

Activating mutations of KRAS have been identified in 10-30% of endometrioid carcinoma (Lax et al., 2000). The KRAS gene encoding the GTPase functions as a molecular switch in intracellular transduction pathway and the mutation in the gene has been associated with tumor growth and differentiation. Mutations are predominantly found in codon 12 and 13 of exon 2 (Kiaris and Spandidos, 1995) and rarely in codon 61 (Semczuk et al., 2001). Moreover, KRAS mutations also detected in endometrial hyperplasia which suggest that mutation in the KRAS gene appears an early event in the development of endometrial carcinoma (Sasaki et al., 1993).

KRAS (Kirsten rat sarcoma viral oncogenes homolog) gene

KRAS gene is a proto-oncogene that belongs to *ras* gene family. The ras family genes consist of three homologue genes (HRAS, KRAS, and NRAS). The KRAS gene is located on the short (p) arm of chromosome 12 at position 12.1. It encodes a 21 kDa guanine nucleotide binding protein (G protein) (Jancík et al., 2010). This gene consists of 6 exons, one 5'non-coding exon and five coding exons (exons 1 to 4A/4B), which exon 4 has 2 alternative spice variants, including KRAS4A and KRAS4B. The KRAS4A isoform includes all coding exons in the mRNA transcript, while KRAS4B excludes exon 4a. Approximately 99% of all KRAS proteins are in the KRAS4B form. The structure of the KRAS gene and its splice variants is shown in Figure 1 (McGrath et al., 1983).

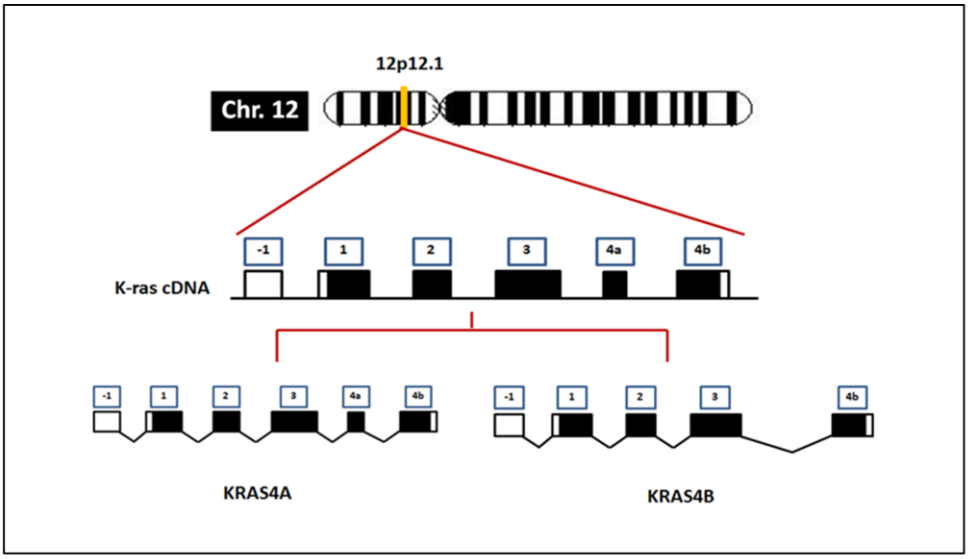


Figure 1. The structure of the KRAS gene and its splice variants. Exons that encode protein are shown as black boxes and non-coding exons as white boxes (Adapted from McGrath et al., 1983).

KRAS Functions and Signaling Pathway

KRAS controls cell proliferation, differentiation, and apoptosis through intracellular signaling transduction by interacting with multiple effectors. KRAS has a GTPase activity and functions as a molecular switch during cell signaling. The GTPase is cycle between active state (ras-GTP) and inactive state (ras-GDP) at the plasma membrane (Jancík et al., 2010). This cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) molecules (Figure 2). KRAS play an important role in RAS-RAF-MEK-ERK signaling pathway, its activation begins when epidermal growth factors (EGF) bind to epidermal growth factor receptor (EGFR) at the cell surface then lead to autophosphorylation of receptors tyrosine kinase. Adaptor proteins such as growth factor receptor bound protein 2 (Grb2) interact with receptor phosphorylated tyrosine, and recruited GEFs such as Son of Sevenless (SOS) to plasma membrane which promote the exchange of GDP to GTP by adding phosphate group to target molecules. And then send the signal through Raf, MEK, and ERK signaling cascade. The ERK molecules stimulate the transcription factors in the nucleus in order to regulate proliferation, survival, and metastasis. The signal is inactivated by GAPs molecules that stimulate hydrolysis of the GTP to GDP (Friday and Adjei, 2005; Mammas et al., 2005). Oncogenic KRAS mutants are resistant to GAPs stimulation. Therefore, the proteins accumulate in the active GTP bound state and constitutively activated (Jancík et al., 2010).

KRAS mutation in Human Cancer

Among RAS genes, KRAS is the most common mutations and has been detected in many types of tumors, approximately 30% of human cancers. KRAS mutations are more frequently found in tumors of pancreas, colorectal, and lung (Fernández-Medarde and Santos, 2011; Kiaris and Spandidos, 1995). Several mutations have been reported in the KRAS gene, however the hotspot codon are codons 12 and 13 of exon 2 which participate in the GTP binding domain of the protein. The alterations in these codons cause their protein impaired to the GTPase activity. Therefore, they persist into an active state and continuously send signals for cell proliferation and differentiation leading to cancer development (Pajkos et al., 2000). Currently, KRAS mutation analysis is used as a predictive marker for EGFR-targeted therapies in both colorectal cancer (CRC) and non-small cell lung cancer (NSCLC) patients. Because activating mutations in this gene at codons 12 and 13 have been associated with lack of response to anti-EGFR therapies.

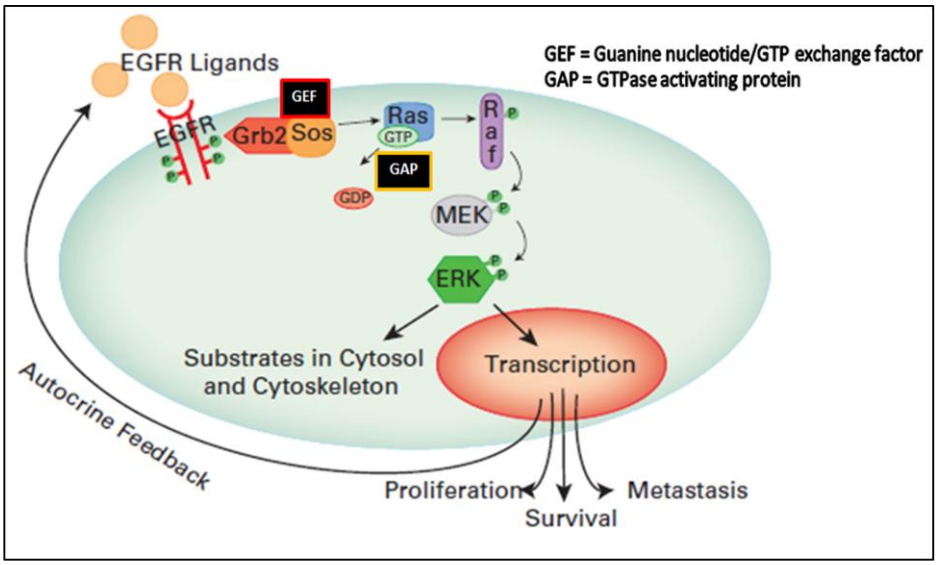


Figure 2. KRAS signaling pathway. Ras proteins are key component of signal transduction pathways leading from cell surface receptors to the control of cell proliferation, survial and metastasis. In active state, Ras-GTP transduces signal pathway of downstream effectors, Raf-MEK-ERK cascade (modified from Roberts et al., 2010).

Mutant KRAS affected to treatment with cetuximab and panitumumab in metastasis CRC patients (Lievre et al., 2006; Amado et al., 2008).

KRAS Mutation in Endometrial Carcinoma

The frequency of somatic KRAS mutation in endometrial carcinoma ranges between 10 and 30% (Kiaris and Spandidos, 1995; Lax 2004). The majority of KRAS mutations are detected in endometrioid types and rarely identified in serous or clear-cell adenocarcinoma (Duggan et al., 1994; Caduff et al., 1995; Lax et al., 2000; Lagarda et al., 2001). Caduff et al (1995) have identified the KRAS mutation at codon 12 in the United States population using amplified created restriction sites (ACRS) method. The mutation was observed in 13 cases (11.6%), consisting of 11 endometrioid carcinomas, one undifferentiated carcinoma, and one carcinosarcoma. The prevalence in this study suggested that KRAS mutations in carcinoma occurred in American less frequency than in Japanese (Caduff et al., 1995). Caduff et al (1995) have shown a significant difference (P=0.02) in the prevalence of the KRAS gene activation between the European/American and Japanese populations. This study suggested that the existence of demographic and/or dietary factors play a role in frequency of the KRAS mutation.

The correlation between the presence of KRAS mutations and clinicopathological parameters has been reported from many studies. Fujimoto et al (1993) have shown that ten of 45 endometrial carcinomas cases were presented KRAS point mutation at codon 12 (22.2%). There was no correlation between the mutation and clinical prognosis, such as clinical stage, histological type, histological grade, depth of myometrial invasion, and ascitic cytology. However, the positive rates of lymph node metastasis tended to be high in the cases with KRAS point mutation. This result is corresponding with the study of Ito et al (1996) who demonstrated the significantly association of KRAS mutations and the presence of lymph node metastasis ($P \leq 0.04$). In addition, they also evaluated the effect of K-*ras* mutation on outcome in patients with different age categories: premenopausal (<53 years), perimenopausal (54-59 years), and postmenopausal (>60 years). KRAS mutation has been found more aggressive clinical behavior in postmenopausal than in premenopausal patients. Alexander-Sefre et al (2003) showed molecular assessment of depth of myometrial invasion basis on KRAS mutation. In contrast, most studies have not found the significant association between KRAS mutations and clinicpathological parameters including age, histological grade, clinical stage, depth myometrial invasion, and lymph node metastasis (Ignar-Trowbridge et al., 1992; Mizuuchi et al., 1992; Caduff et al., 1995). Mizuuchi et al

(1992) found no relationship of KRAS mutation and age of patients, clinical stage, and depth of myometrial invasion. This result indicated that KRAS activation is an independent risk factor in endometrial carcinoma.

Materials and Methods

Patients

A retrospective cohort of 190 women who underwent hysterectomy for endometrial cancer between 2008-2012 was assembled with approval from the Hospital Ethics Committee of Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand. All patients were diagnosed as endometrioid adenocarcinoma of uterus. Clinical and pathological data for the cohort were collected from patient records.

DNA extraction and PCR analysis

A total of 190 formalin-fixed paraffin-embedded tumor samples were selected for KRAS analysis after reviewing the hematoxylin & eosin (H&E)-stained slides. Tumor areas on the H&E-stained slide were identified and were marked and microdissected with a blade on a corresponding unstained slide. The tumor tissues were transferred to an Eppendorf tube for subsequent DNA isolation. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The PCR for KRAS in exon 2 was performed using two primer set. The primers for the 291 bp amplicon were F1:5'-GTACTGGTGGAGTATTTGAT-3', and R1:5'ACTCATGAAAATGGTCA GAG-3'. The primers for the shorter 214 bp amplicon were F2: 5'-CTTATGTGTGACAT GTTCT-3' and R2: 5'-AGAATGGTCCTGCACCAGTA-3'. All samples were screened for the first PCR amplification with F1, R1 primer pair. DNA samples that were negative by primers F1, R1 were re-amplified using 2 µl of the first PCR product as a template for the nested PCR. The nested PCR was performed with primers F2, R2. The quality of DNA was amplified with primer of beta globin gene. The beta globin primers for the 268 bp amplicon were PC04:5'-CAACTTCATCCACGTTCACC-3', and GH20:5'-GAAGAGCC AAGGACAGGTAC-3'. Each PCR reaction was performed in a 25 µl of total volume containing 1x PCR buffer with 2 mM $MgCl_2$, 200 µM of dNTPs, 0.2 µM of each primer, 1 unit of FastStart Taq DNA polymerase (Roche, Germany), 50-500 ng of genomic DNA, and distilled water. The PCR reaction was amplified with initial denaturation at 94°C for 4 minutes, followed by 40 cycles of denaturation 94°C for 45 seconds, annealing at 51 °C (KRAS), and 55 °C (beta-globin) for 45 seconds, and extension at 72 °C for 1 minute and with final extension at 72 °C for 7 minutes. The PCR products were run on a 2% agarose gel electrophoresis and stained with ethidium bromide for size verification.

DNA sequencing

PCR products were purified using Purelink PCR Purification kit (Invitrogen, Germany) according to the manufacturer's instructions. The purified PCR products were eluted in a 15 µl volume and were quantified using nanodrop spectrophotometer. The purified PCR product was then used as template in cycle sequencing with the BigDye Terminator v1.1 kit (Applied Biosystems, USA).The sequencing PCR product was precipitated using ethanol and run on a ABI PRISM 310 sequencer (Applied Biosystems). Sequencing data was analyzed using sequencing analysis 3.7 software. Electropherogram data were evaluated.

Reverse dot blot hybridization

Amino modified oligonucleotides were synthesized as probes targeting 7 mutations in codon 12, 13, and 33 of the K-ras gene. An additional oligonucleotide of wild type in codon 12 and 13 was included. All probes are listed in Table 1. Subsequently, 1 µl of each 2 pmole oligonucleotide probes was spotted onto activated Biodyne C membrane sheets and the sheets were then cut to define test strip 5 mm wide. The biotinylated PCR products were hybridized to membrane test strips strictly controlling temperature (48°C) for 40 min. After washing, the bound sequences were visualized using a streptavidin-alkaline phosphatase conjugate and color substrates by a mixture of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyphosphatase (BCIP) according to protocol. The presence of blue staining in a clear background was interpreted as positive.

Statistical analysis

Statistical analysis was performed using R program. The relationship between clinicopathological variables and the presence of KRAS mutation was determined using χ^2 (chi-square) test. Statistically significant was set at $P < 0.05$.

Table 1. Primers and oligonucleotide probes for KRAS mutations using reverse dot blot hybridization

Primer & Probe	Sequence 5′-3′	Reference
1. Primer Ras-F	5-GGC CTG CTG AAA ATG ACT GAA-3	Ausch et al.,2009
2. Primer Ras-R	5-biotin-TTC GTC CAC AAA ATG ATT CTG A-3	
3. Probe-Ras-w	5-NH_2- GGA GCT GGT GGC GTA GGC-3	
4. Probe-Asp12	5-NH_2- GGA GCT GAT GGC GTA GGC-3	
5. Probe-Val12	5-NH_2- GGA GCT GTT GGC GTA GGC-3	
6. Probe-Ala 12	5-NH_2- GGA GCT GCT GGC GTA GGC-3	
7. Probe-Arg 12	5-NH_2- GGA GCT CGT GGC GTA GGC-3	
8. Probe-Cys12	5-NH_2- GGA GCT TGT GGC GTA GGC-3	
9. Probe-Ile 12	5-NH_2- GGA GCT ATT GGC GTA GGC-3	
10. Probe-Leu 12	5-NH_2- GGA GCT CTT GGC GTA GGC-3	
11. Probe-Ser12	5-NH_2- GGA GCT AGT GGC GTA GGC-3	
12. Probe-Asp13	5-NH_2- GGA GCT GGT GAC GTA GGC-3	
13. Probe-Cys13	5-NH_2- GGA GCT GGT TGC GTA GGC-3	

Results

Of the 190 patients with endometrioid endometrial adenocarcinoma, the median age of patients was 57 years, with a range from 26 to 86 years. Sixteen patients (8.4%) were age ≤ 40 years. One hundred and ten (57.9%) were age > 40- ≤ 60 years. Sixty four patients (33.7%) were in postmenopausal period (> 60 years). Sixty patients (31.6%) were nulliparous while 130 patients (68.4%) were multiparous. Of all patients investigated, the BMI ranged from 17 to 51 kg/m^2 (median BMI=27.4 kg/m^2). Seventy six patients (40%) were overweight (BMI =25-30 kg/m^2), and 46 patients (24.2%) met the criteria of obesity (BMI > 30 kg/m^2). Fifty two patients (27.4%) had diabetes mellitus and 87 patients (45.8%) had hypertension. One hundred fifty seven patients (82.6%) had grade I-II tumors while 33 (17.4%) had grade III tumor. The distribution by FIGO stage was: stage I, 138 (72.6%); II, 19(10%); III, 30 (15.8%); and IV, 3 (1.6%). One hundred seventy eight patients (93.7%) had undergone lymph node dissection or sampling, and 21 patients (11.1%) showed lymph node involvement. Synchronous ovarian cancer/ovarian metastasis was found in 9 patients (4.7%). Clinicopathological characteristics of the patients are summarized in Table 2.

KRAS Mutations by direct sequencing

Of all cases investigated, KRAS mutations were detected in 37 of 190 cases (19.5%), shown in Table 3. In particular, 33 of the 190 cases (17.4%) were amino acid substitutions (missense mutation), whereas 4 of the 190 cases (2.1%) showed silent change. All missense mutation, 57.6% (19 of 33) were found in codon 12 and 18.2% (6 of 33) in codon 13 (19 of 33 tumors presented single point mutations, and 5 of 33 presented double point mutations in codon 12 with other codons: 2, 6, 7, and 13) (Figure 3). There were 9 cases of missense mutations detected at other sites of exon 2 including codon 3, 14, 15, 27, 29, 33, and 34. All 9 cases showed single point mutations.

The most frequent mutation in codon 12 was glycine to aspartate (GGT to GAT), which was detected in 11 of 33 cases (33.3%). The other mutations observed in codon 12 resulted in replacement of glycine with serine (GGT to AGT; 1 case, 3%), cysteine (GGT to TGT; 4 cases, 12.1%), and valine (GGT to GTT; 3 cases, 9.1%). The most frequent mutation in codon 13 was glycine to aspartate (GGC to GAC), which was present in 5 of 33 cases (15.2%). The other mutation was glycine to cysteine (GGC to TGC) in one of 33 cases (3%). The frequency of missense mutations identified in endometrioid adenocarcinoma is summarized in Table 4. Overall, the type of mutation was G:C to A:T transitions (29 of 33, 87.9%) at high frequency than G:T transversions (8 of 33, 24.2%).

The correlation between K-ras mutation and clinicopathological parameters

No significant relationship was seen between the presence of KRAS mutations and clinicopathological variables (age, BMI, parity, grade, stage and depth of myometrial invasion, LVSI, uterine cervical and lymph node involvement, and synchronous ovarian cancer/ovarian metastasis) analyzed as shown in Table 5. With respect of prognosis, 3 out of 33 patients with KRAS missense mutations died of cancer, whereas, 10 out of 153 patients without KRAS mutation died of cancer.

Reverse dot blot hybridization

Reverse dot blot hybridization was tested in 30 selected samples. Four of the 30 were interpreted as KRAS wild type, whereas 26 of the 30 showed KRAS mutations. The mutations were reported on codon 12, namely Gly12Asp (11 cases, 42.3%), Gly12Cys (4 cases, 15.4%), Gly12Ser (1 case, 3.8%), and Gly12Val (3 cases, 11.5%); on codon13, namely Gly13Asp (4 cases, 15.4%), and Gly13Cys (1 case, 3.8%); only 2 mutations (7.7%) were reported on codon33, both Asp33Gly (Figure 4).

Concordance of Reverse dot blot hybridization and direct sequencing

Detecting all of K-ras mutations, the 2 methods were found to have the same results (Table 6, Figure 5).

Table 2. Clinical and pathological characteristics of 190 patients with endometrioid adenocarcinoma of endometrium

Characteristic		No. of patients (%)
Age (years)		
	≤40	16 (8.4)
	>40 - ≤60	110 (57.9)
	>60	64 (33.7)
BMI (kg/m^2)		
	<25	68 (35.8)
	≥25-30	76 (40)
	>30	46 (24.2)
Parity		
	Nulliparous	60 (31.6)
	Multiparous	130 (68.4)
DM		
	No	138 (72.6)
	Yes	52 (27.4)
HT		
	No	103 (54.2)
	Yes	87 (45.8)
Tumor grade		
	grade I	98 (51.6)
	grade II	59 (31)
	grade III	33 (17.4)
Stage		
	I	138 (72.6)
	II	19 (10)
	III	30 (15.8)
	IV	3 (1.6)
Myometrial invasion		
	No	25 (13.1)
	<50%	94 (49.5)
	≥50%	71 (37.4)
LVSI		
	No	140 (73.7)
	Yes	50 (26.3)
Uterine cervical involvement		
	No	175 (92.1)
	Yes	15 (7.9)
Lymph node involvement		
	No	157 (82.63)
	Yes	21 (11.1)
	∗none	12 (6.31)
Synchronous Ovarian cancer/ Ovarian metastasis		
	No	181 (95.3)
	Yes	9 (4.7)

∗ none = no lymph node

Table 3. Patterns of KRAS mutation status in exon 2 detected in 37 patients with endometrioid endometrial adenocarcinoma

KRAS Codon	Mutation type	Nucleotide alterations	Amino acid alterations	frequency (%)
hotspots				
12	Missense	GGT>AGT (12-1-A)	Gly>Ser	1
12	Missense	GGT>TGT (12-1-T)	Gly>Cys	2
12	Missense	GGT>GAT (12-2-A)	Gly>Asp	8
12	Missense	GGT>GTT (12-2-T)	Gly>Val	3
			Total	14 (41.7)
13	Missense	GGC>TGC (13-1-T)	Gly>Cys	1
13	Missense	GGC>GAC (13-2-A)	Gly>Asp	4
			Total	5 (13.9)
12,2	Missense	GGT>GAT (12-2-A)	Gly>Asp	1
	Missense	ACT>ATT (2-2-T)	Thr >Ile	
12,6	Missense	GGT >TGT (12-1-T)	Gly>Cys	1
	Missense	TTT >CTT (6-1-C)	Phe>Leu	
12,7	Missense	GGT>GAT (12-2-A)	Gly>Asp	1
	Missense	GTG >ATG (7-1-G)	Glu>Met	
12,13	Missense	GGT >TGT (12-1-T)	Gly>Cys	1
	Missense	GGC >GAC (13-2-A)	Gly>Asp	
12,13	Missense	GGT>GAT (12-2-A)	Gly>Asp	1
	Silent	GGC>GGT (13-3-T)	Gly>Gly	
			Total	5 (11.1)
Others				
3	Missense	GAA>AAA (3-1-A)	Glu>Lys	1
3	Missense	GAA>GGA (3-2-G)	Glu>Gly	1
11	Silent	GCT >GCC (11-3-C)	Ala> Ala	1
14	Missense	GTA>ATA (14-1-A)	Val>Ile	1
15	Missense	GGC>AGC (15-1-A)	Gly>Ser	1
16	Silent	AAG>AAA (16-3-A)	Lys>Lys	1
25	Slient	CAG>CAA (25-3-A)	Gln>Gln	1
27	Missense	CAT>TAT (27-1-T)	His>Tyr	1
29	Missense	GGA>GAA (29-2-A)	Gly>Glu	1
33	Missense	GAT>GGT (33-2-G)	Asp>Gly	2
34	Missense	CCA>TCA (34-1-T)	Pro>Ser	1
19,22	Silent	TTG>TTA (19-3-A)	Leu>Leu	1
	Silent	CAG>CAA (22-3-A)	Gln>Gln	
			Total	13 (33.3)
			All	37

Table 4. Patterns of KRAS missense mutations detected in 33 patients with endometrial endometrioid adenocarcinoma

KRAS	Nucleotide alteration	Mutation transitions	Mutation transversions	Frequency
Codon 12	GGT>TGT (12-1-T)		G :T	2
	GGT>AGT (12-1-A)G : A		1	
	GGT>GAT (12-2-A)G : A		8	
	GGT>GTT (12-2-T)		G : T	3
Codon 12 & 2	GGT >GAT (12-2-A)G : A		1	
	ACT >ATT (2-2-T)	C : T		1
Codon 12 &6	GGT >TGT (12-1-T)		G : T	1
	TTT >CTT (6-1-C)	T : C		1
Codon 12&7	GGT>GAT (12-2-A)G : A		1	
	GTG >ATG (7-1-G)	G : A		1
Codon 12&13	GGT>TGT (12-1-T)		G : T	1
	GGC >GAC (13-2-A)	G : A		1
Codon 12&13	GGT>GAT (12-2-A)G : A		1	
	GGC >GGT (13-3-T)			
Codon 13	GGC>TGC (13-1-T)		G : T	1
	GGC>GAC (13-2-A)	G : A		4
Codon 3	GAA>AAA (3-1-A)	G : A		1
	GAA>GGA (3-2-G)	A : G		1
Codon 14	GTA>ATA (14-1-A)	G : A		1
Codon 15	GGC>AGC (15-1-A)	G : A		1
Codon 27	CAT>TAT (27-1-T)	C : T		1
Codon 29	GGA>GAA (29-2-A)	G : A		1
Codon 33	GAT>GGT (33-2-G)	A : G		2
Codon 34	CCA>TCA (34-1-T)	C : T		1
			Total	37

Table 5. Correlation between clinicopathological features and KRAS mutation status (missense type) in 190 endometrial endometrioid adenocarcinomas

	Total (%)	KRAS mutations positive (%)	KRAS mutation negative (%)	*p*-value
All patients	190 (100)	33 (17.4)	157 (82.6)	
Age (yrs)				
Range	26-86	27-86	26-79	
Average age	56.1	56.4	56.1	
≤60	126	23 (18.3)	103 (81.7)	0.49
>60	64	10 (15.6)	54 (84.4)	
BMI				0.827
<25	68	14 (20.6)	54 (79.4)	
≥25-30	78	13 (16.7)	65 (83.3)	
>30	44	6 (13.6)	38 (86.4)	
Parity				0.596
Nulliparous	60	11 (18.3)	49 (81.7)	
Multiparous	130	22 (16.9)	108 (83.1)	
DM				0.167
No	138	26 (18.8)	112 (81.2)	
Yes	52	7 (13.5)	45 (86.5)	
HT				0.61
No	103	18 (17.5)	85 (82.5)	
Yes	87	15 (17.2)	71 (82.8)	
Tumor grade				0.226
grade I	98	19(19.4)	79 (80.6)	
grade II	59	12 (20.3)	59 (79.7)	
grade III	33	2 (6.1)	31 (93.9)	
Stage				0.171
I	138	29 (21)	109 (79)	
II	19	2 (10.5)	17 (89.5)	
III & IV	33	2 (6.1)	31 (93.9)	
Myometrial invasion				0.952
No	25	4 (16)	21 (84)	
<50%	94	15 (16)	79 (84)	
≥50%	71	14 (19.7)	57 (80.3)	
LVSI				1.0
No	140	24 (17.1)	116 (82.9)	
Yes	50	9 (18)	41 (82)	
Uterine cervical involvement				0.501
No	175	31 (17.7)	144 (82.3)	
Yes	15	2 (13.3)	13 (86.7)	
Lymph node involvement				0.495
No	157	31 (19.7)	126 (80.3)	
Yes	21	2 (9.5)	19 (90.5)	
None	12	0	12 (100)	
Synchronous Ovarian cancer/Ovarian metastasis				0.821
No	181	32(17.7)	149 (82.3	
Yes	9	1 (11.1)	8 (88.9)	

Table 6. Concordance of KRAS mutations in 30 samples using reverse dot blot hybridization and direct sequencing

No	reverse dot blot hybridization	direct sequencing
1	G12D	G12D
2	G12D	G12D
3	G12D	G12D
4	G12D	G12D
5	G12D	G12D
6	G12D	G12D
7	G12D	G12D
8	G12D	G12D
9	G12D	G12D
10	G12D	G12D, G13G
11	G12D	G12D, V7M
12	G12C	G12C
13	G12C	G12C
14	G12C	G12C, F6L
15	G12C, G13D	G12C, G13D
16	G12S	G12S
17	G12V	G12V
18	G12V	G12V
19	G12V	G12V
20	G13D	G13D
21	G13D	G13D
22	G13D	G13D
23	G13D	G13D
24	G13C	G13C
25	D33G	D33G
26	D33G	D33G
27	12,13WT	12,13WT
28	12,13WT	12,13WT
29	12,13WT	12,13WT
30	12,13WT	12,13WT

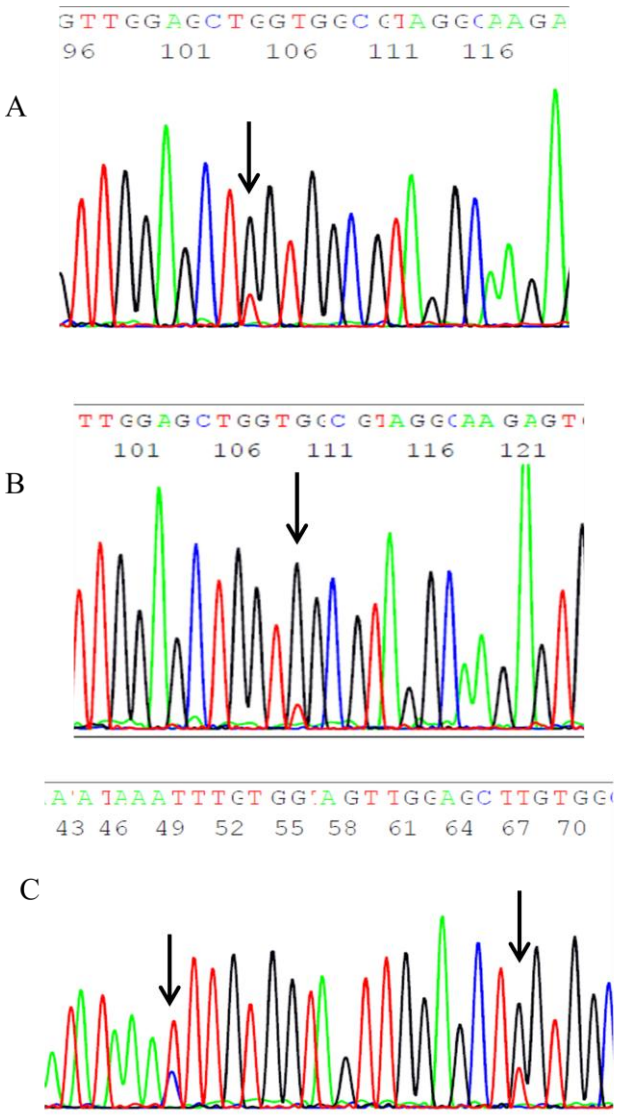


Figure 3. Sequencing analysis for exon 2 of the KRAS mutations in endometrioid adenocarcinomas A. point mutation at codon12 (GGT> TGT), B. at codon 13(GGC>TGC), C. at codon 6 (TTT>CTT) and 12 (GGT>TGT)

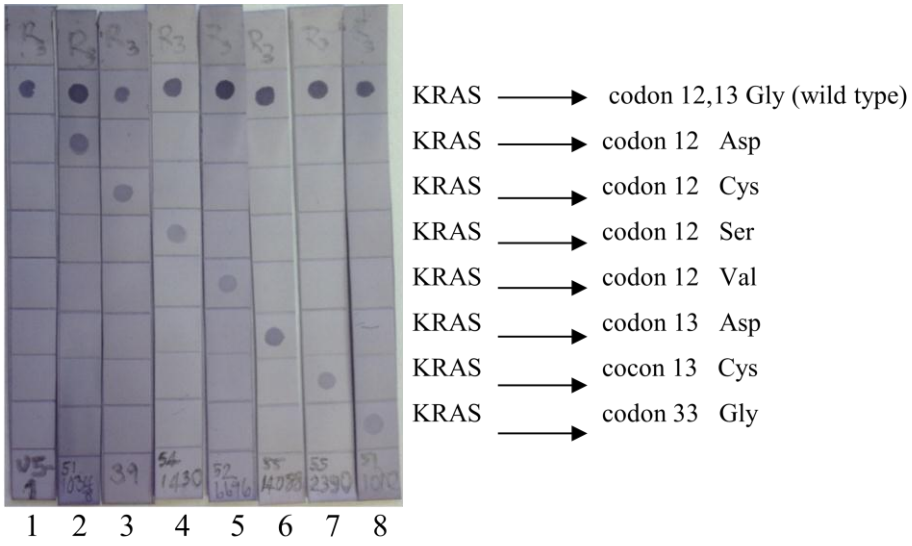
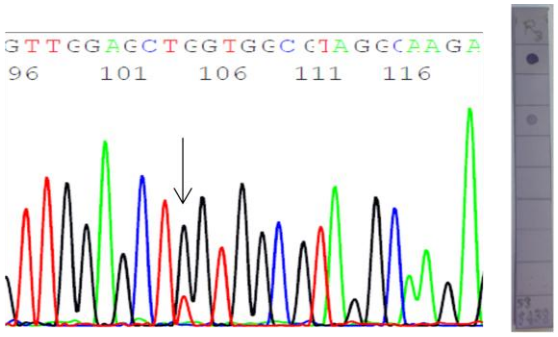
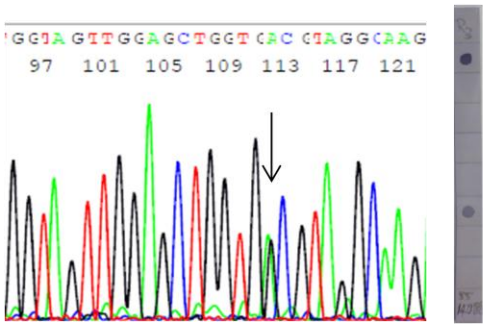


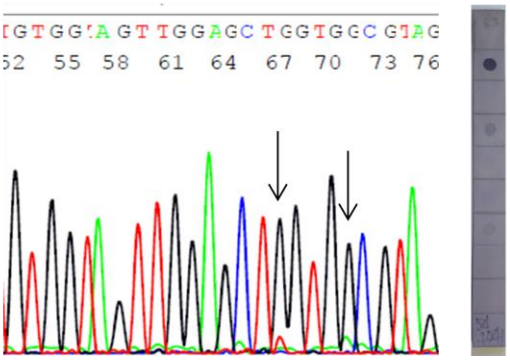
Figure 4. Reverse dot blot hybridization analysis from 8 tumor samples showing KRAS mutation on codon 12,13, and 33. No1. negative for KRAS mutation on codon 12 and 13 (Gly-Gly), No 2-5. presence of KRAS mutation on codon12 (Gly>Asp-Cys-Ser-Val), No 6-7. presence of KRAS mutation on codon 13 (Gly>Asp-Cys), No 8. presence of KRAS mutation on codon 33 (Asp>Gly), respectively.



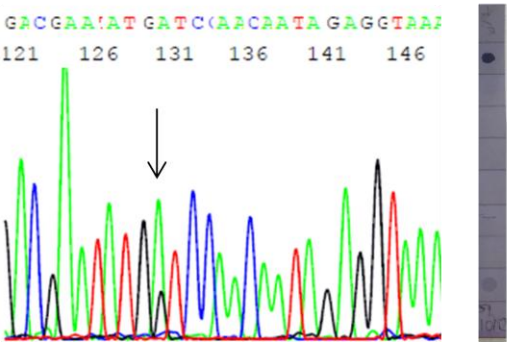
A. codon 12 (GGT>TGT, Gly>Cys).



B. codon 13 (GGC>GAC, Gly>Asp).



C. codon 12 (GGT>AGT, Gly>Ser) and codon 13 (GGC>GAC, Gly>Asp).



D. codon 33 (GAT>GGC, Asp>Gly).

Figure 5. Concordance of KRAS mutations using direct sequencing and reverse dot blot hybridization. A shows mutation at codon 12 (GGT>TGT). B shows mutation at codon 13 (GGC>GAC). C shows mutation at codon12 and 13 (GGT>TGT and GGC>GAC). D shows mutation at codon 33 (GAT>GGT)

Discussion

Endometrial cancer is the third most common gynecologic cancers in Thailand. The majority of tumors is histolocially diagnosed as the endometrioid type. It has been reported that obesity was only the independent factor associated with endometrial cancer in Thai patients aged 45 years or younger (Hanprasetpong et al., 2008 and Manchana and Khemapech, 2008). The molecular basis of carcinogenesis in endometrial cancer Thai patient was still little studied. Several reports have demonstrated a number of molecular alterations in endometrial cancer, such as PTEN inactivation, loss of FOXO1 expression, amplification and overexpression of HER-2, overexpression of p53, and mutation in KRAS (Ellis et al., 2000). The KRAS proto-oncogene is associated with changes increased cell proliferation and inhibition of apoptosis. Mutations of KRAS gene have been detected in several types of tumors including carcinomas of pancreas, colon, lung, and endometrium. Our study showed that the frequency of mutations in exon 2 of KRAS gene in endometrioid adenocarcinoma was 17.4% (33/190). This finding is in line with previous studies, in which the frequency ranges from 10 to 30% (Mizuuchi et al., 1992, Llobet et al., 2009, Dobrzycka et al., 2009). The most common location of point mutations occurred in K-ras codon 12 rather than codon 13 (Ito et al., 1996, Semczuk et al., 1998). Our results showed KRAS mutation in exon 2 located at codon 12 (10%), codon13 (3.2%), and other sites (4.7%). KRAS codons 12 and 13 coding for two adjacent glycines are the most common sites of oncogenic activation (Edkins et al., 2006). Any mutation resulting in amino acid alterations at these codons, which encodes amino acids adjacent to the GDP/GTP binding pocket, reduces or abolishes GTPase activity of KRAS after GAP binding and locks the protein in an active, GTP-bound state (Seeburg et al., 1984). In accordance with our data, previous studies have usually identified the incorporation of other amino acids, most commonly aspartate and valine at codon 12 and aspartate at codon 13 (Andreyev et al., 1998, Neumann et al., 2009, Byron et al., 2012, and Xiong et al., 2013). These amino acids alterations cause projection of larger amino acid side chains into the GDP/GTP binding pocket of the protein that interfere with geometry of the transition state in which GTP hydrolysis is catalysed (Malumbres and Barbacid, 2003).

Interestingly, among 19 missense mutations of KRAS gene located at codon12 in our study, the presence of double mutations was detected in 5 samples, recoding KRAS codons 12 and 2 (G12D/T2I); codons 12 and 6 (G12C/F6L); codons 12 and 7 (G12D/E7M); and codons 12 and 13 (G12C/G13D). These double mutations of KRAS codon 12 and codons 2, 6, and

7, respectively, have not been previously reported. Additionally, the KRAS mutation at codons 12 and 22 occurring in the same allele was identified in a case of colon cancer (Miyakura et al., 2002). It has been reported a double mutation of KRAS codon 19 (leucine to phenylalanine, L19F) and codon 20 (threonine to alanine, T20A) in a colorectal cancer (Naguib et al., 2011). This co-mutation of the KRAS L19F/T20A was shown to the synergistic effects on transformation in vitro.

Our present study showed the KRAS point mutation sites outside of codons 12 and 13. They were located at codons 3, 14, 15, 27, 29, 33, and 34. Previous studies have reported that the mutational sites were not only located on hot spot codons 12 and 13, but they were also located at codons 15, 18, 20, 27, 30, and 31 in colorectal cancerous tissues and adrenal tumors (Lin et al., 1998, Wang et al., 2003, and Yen et al., 2010). The mutation of KRAS at codon 15 decreased the binding ability of the mutant KRAS protein to GTPase activation proteins. However, the exact mechanism of any KRAS mutation outside codon hot spots in cancerous tissues remains largely unknown.

Point mutations in KRAS gene are relatively found in atypical endometrial hyperplasia, suggesting they are early events in endometrial carcinogenesis (Sasaki et al., 1993, Dobrzycka et al., 2009). Additionally, the frequency of KRAS mutations has been reported in endometrial carcinomas with various histological types (Sasaki et al., 1993, Pappa et al., 2006, Alexander-Sefre et al., 2003, and Xiong et al., 2013). The prognostic impact of KRAS mutational status in endometrial cancers has been inconsistent. Ito et al (1996) showed the presence of KRAS mutation in endometrioid cancers was significantly associated with lymph node metastases and poor survival among patients in ages above 60 years. A borderline significant correlation between presence of submicroscopic myometrial invasion and depth of myometrial invasion was observed in endometrial cancer patients with stage I (P = 0.053) (Alexander-Sefre et al., 2003). The presence of KRAS mutations was significantly associated with endometrial subtype, low grade and obesity, but was not correlated with metastatic lesions and clinical outcome (Birkeland et al., 2012). Our findings showed that KRAS mutations occurred mostly in patients with grade I- II and stage I-II, however, no significant correlation was noted. In agreement with previous studies, KRAS mutations was not related to any pathological, histological, or clinical parameters (Ignar-Trowbridge et al., 1992, Semczuk et al., 1998, Pappa et al., 2006).

Comparing between direct sequencing and reverse dot blot hybridization, the concordance of mutation detection was 100%. Although DNA sequencing remains the gold standard for

identification of specific mutations, reverse dot blot hybridization assay described here is effective and sensitive technique for detection of mutations in codons 12, 13, and 33 of k-ras gene. The procedure seems to be fast and easy to follow a simple protocol using ready to use test strips and visible color detection. Moreover, this assay also has the advantage of using short DNA fragment (less than 150 bp) isolated from samples of FFPE tissues to be amplified by PCR.

In conclusion, this study provides preliminary evidence that the presence of KRAS mutations may be a relatively common event in endometrial carcinogenesis in subgroup of Thai patients, but their prognostic value is limited. With an alternative tool applicability comparing to direct sequencing, reverse dot blot hybridization in our study is an effective and reliable assay for screening of KRAS point mutations in FFPE tissues.

Conclusions

This research provides preliminary evidence to report the prevalence of KRAS mutations and their correlation with the clinicopathological parameters in Thai patients with endometrial endometrioid adenocarcinoma. In addition, with alternative tool applicability, reverse dot blot hybridization technique is an effective and reliable to identify the KRAS point mutation in FFPE tissues.

The main findings of this study are concluded as followings:

1. The prevalence of KRAS mutations in Thai patients with endometrial endometrioid adenocarcinoma was 19% (37 of 190) and the mutations were predominantly found in codons 12 and 13 of the KRAS exon 2.

2. The novel mutational sites of KRAS exon 2 were reported in this study, including codons 3, 14, 27, 29, 33, and codon 34.

3. KRAS gene mutations were associated with the development of malignancy in subgroup of Thai endometrial cancer patients but their prognostic value was limited.

4. Reverse dot blot hybridization assay appears to be effective and reliable method for detecting KRAS mutation in DNA isolated from FFPE tissue samples.

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