Novel approach to noninvasive assessment of lymph nodes metastases with one-step isothermal quantification of mRNAs in primary tumor of endometrial cancer

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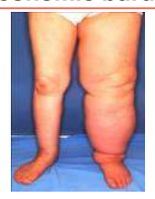
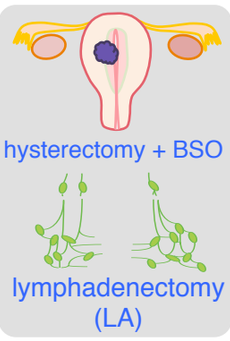
Background: Lymphadenectomy in endometrial cancer should be considered depending on individual patients owing to its postoperative risks such as lymphedema. Although assessment of lymph node metastasis provides crucial information for appropriate adjuvant management, therapeutic importance of lymphadenectomy for low-intermediate risk patients is a matter of debate. Given that sentinel lymph node biopsy is a beneficial but complex method, noninvasive, simple and high-precision diagnosis method for lymph node metastatic state is highly demanded. In a previous study, we identified SEMA3D and a novel isoform of TACC2 as promising biomarkers to evaluate lymphatic metastasis based on gene expression patterns in the primary lesion. Here, we attempted to optimize sampling method considering tumor heterogeneity and to accelerate gene quantitative analysis for our biomarkers.
Methodology: Endometrial cancer tissues from new several patients were collected in addition to the previous 115 patients. We collected 5-13 pieces of primary tumor from each new patient. We verified gene expressions of each tissue piece by quantitative-PCR to optimize sampling method. Then we assessed whether RT-SmartAmp method, which can detect nucleic acids in one step consisting of a reverse transcription and an isothermal amplification of DNA, could quantify our biomarker RNA rapidly. We measured the speed of amplification and target specificity based on biomarker RNA as the template in one step containing reverse transcription step.
Results: We calcu lated the average expression levels by bootstrap method It suggested that even when the sampling number is two, each average expression level indicates correct diagnosis with 99%CI. Then we also succeeded to develop a promising candidate of primer set to detect the target mRNA by using SmartAmp. This candidate can detect RNA quantitatively within 30 minutes without non-specific amplification of negative control by using Eprimer as detection fluorescence.
Conclusion: Our finding s pave the way for support clinical decisions that minimize irrelevant lymphadenectomy.

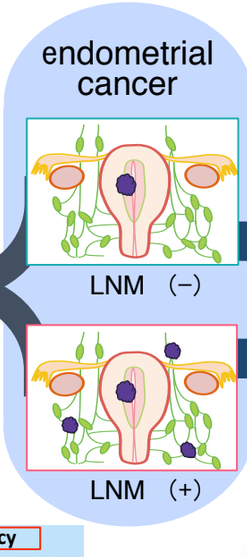
present

deterioration of postoperative QOL
- Invasion of surgery
- Physical burden
- Mental burden
- Economic burden



systematic treatment





gene expression difference

differential analysis of 5,0000 gene expression

The most significant 2 genes were identified.

SEMA3D — High expression in LNM(−)

novel TACC2 isoform — High expression in LNM(+)

future plan

Selective LA approach; LA is omitted in LNM(−) and added in LNM(+).

KCC risk-scoring system

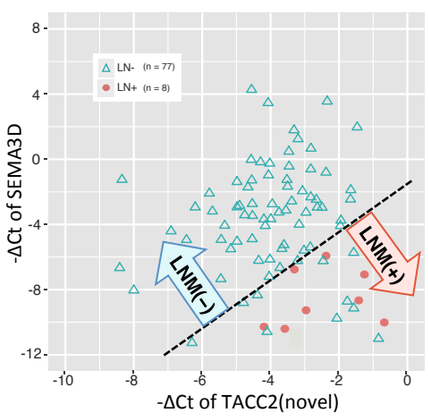
MRI Tumor Volume 6cm³ ≦	1point
CA125 25 U/ml < (pre-menopose) / 70 U/ml < (post-menopose)	1point
Tumor grade/histology except G1	1point
Myoinvasion 1/2 <	1point

+ collect primary tumor tissue → gene expression analysis → diagnosis of LNM → selective LA approach (during operation)

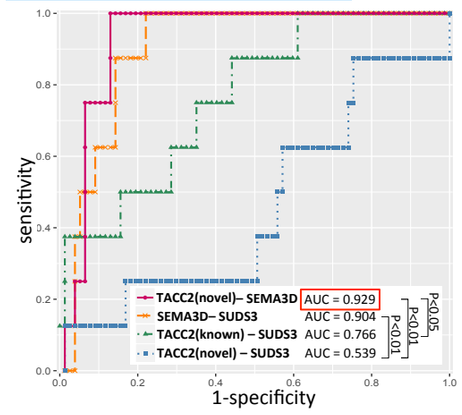
LNM(−) → Minimallized invasive surgery: hysterectomy + BSO only

LNM(+) → adequate surgery: hysterectomy + BSO + lymphadenectomy

Intraoperative high accuracy rapid diagnosis kit
- rapid nucleic quantitative analysis
- Prediction system by risk-scoring



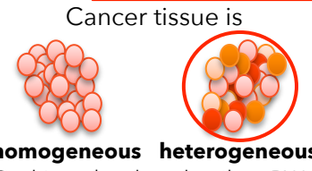
AUC 0.9 - 1.0	High accuracy
AUC 0.9 - 0.7	Moderate accuracy
AUC 0.5 - 0.7	Low accuracy



Aim 1

Acceleration of gene quantitative analysis for our biomarkers

Cancer tissue is

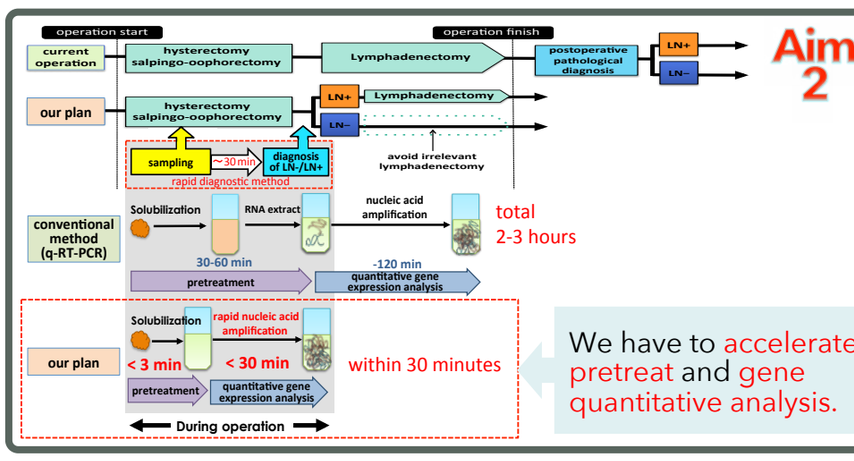


homogeneous **heterogeneous**

Our biomarkers based on the mRNA expression levels of primary region.

How should we collect samples for accurate diagnosis from heterogeneous cancer tissue?

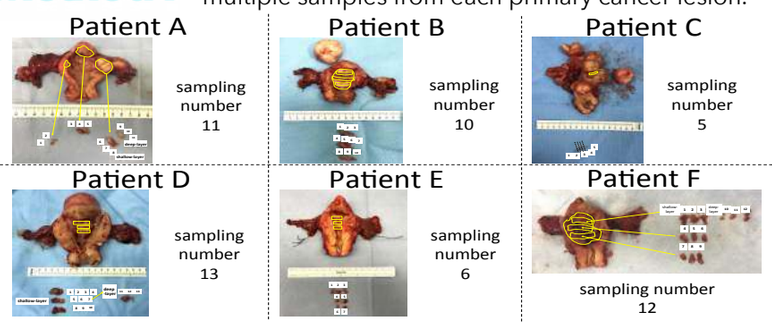
Aim 2



We have to accelerate pretreat and gene quantitative analysis.

Method1

We chose 6 patients at random and collected multiple samples from each primary cancer lesion.



RNA extraction → cDNA synthesis → qRT-PCR

each Sample

Total RNA was extracted and isolated from each samples RNeasy® Plus Mini Kit (Qiagen)

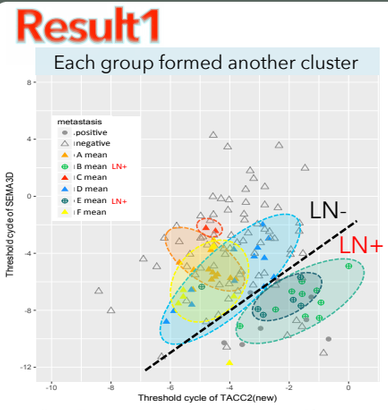
2μg of total RNA was reverse-transcribed with PrimeScript® 1st strand cDNA Synthesis Kit (TaKaRa)

To validate the expression levels of biomarker genes, qPCR was carried out with ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

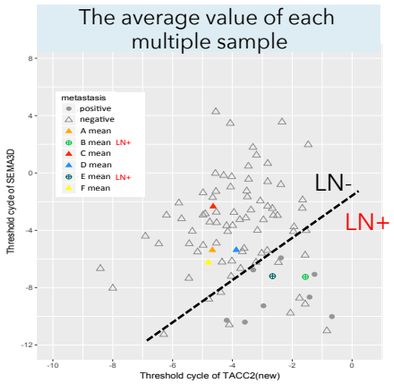
The analysis data was compared with previous ones.

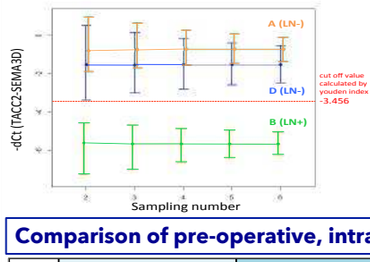
Result1

Each group formed another cluster



The average value of each multiple sample





We selected 100,000 times in each case by bootstrap method and calculated the average expression level. Error bars show 99% confidence interval of the average value. Even when the sampling number is two, each average expression level is not overlapped and indicates correct diagnosis. In consideration of sampling error, it has to be collected 3 different samples from each primary lesion.

Comparison of pre-operative, intra-operative and post-operative diagnosis

	pre-operative diagnosis		post-operative diagnosis		our biomarker
patient	pathology	lymph node metastasis by diagnostic imaging (PET/CT etc)	pathology	lymph node metastasis by pathological diagnosis	lymph node metastasis
A	G1	LN-	G1	LN-	LN-
B	G1	LN-	G2	LN+	LN+
C	G1	LN+	G1	LN-	LN-
D	G3	LN+	G3	LN-	LN-
E	G1	LN+	G1	LN+	LN+
F	G1	LN+	G1	LN+	LN+

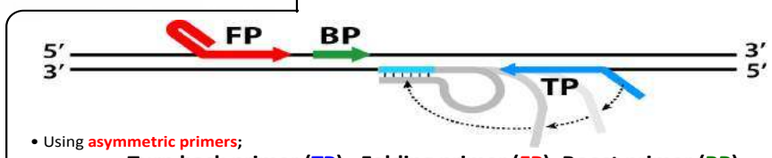
Final diagnosis of lymph node metastatic state are based on post operative diagnosis.
Our biomarker predicted each lymph node metastatic state correctly in all cases.

diagnostic imaging	LN+	LN-	total
Metastasis positive	3	4	7
Metastasis negative	False Negative 5	73	78
Total	8	77	85
Sensitivity			37.5%
Specificity			94.8%
Positive Predictive Value, PPV			42.8%
Negative Predictive Value, NPV			93.5%

our biomarker	LN+	LN-	total
Metastasis positive	8	10	18
Metastasis negative	False Negative 0	67	67
Total	8	77	85
Sensitivity			100%
Specificity			87.0%
Positive Predictive Value, PPV			44.4%
Negative Predictive Value, NPV			100%

Method2

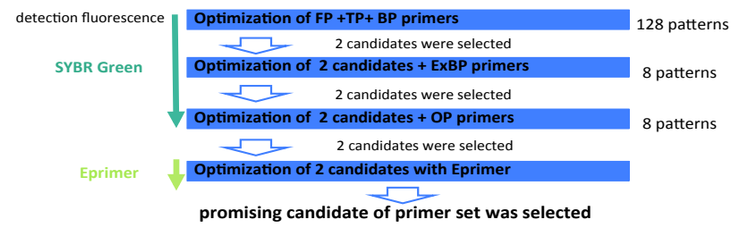
SmartAmp: Smart Amplification Process



- Using asymmetric primers;
  Turn back primer (TP) , Folding primer (FP), Boost primer (BP)
- Using **Strand-displacement** DNA polymerase.
  "Aac DNA polymerase I"
- Simple incubation (**60°C isothermal**).
- Rapid DNA amplification (positive detection point: **15-30 minutes**).
- 1st Reverse-transcription (**RT**) can be combined for **RNA detection** in one step reaction.

(1) We desgined a set of primers for SmartAmp across the exon-exon junction of the novel isoform of TACC2, in order to avoid amplification of genomic DNA.

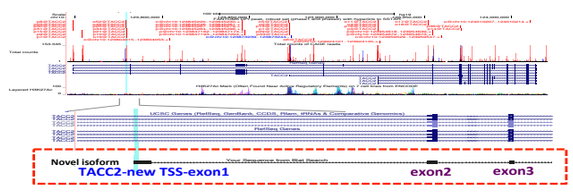
(2) We measured the speed of amplification and target specificity based on cDNA.

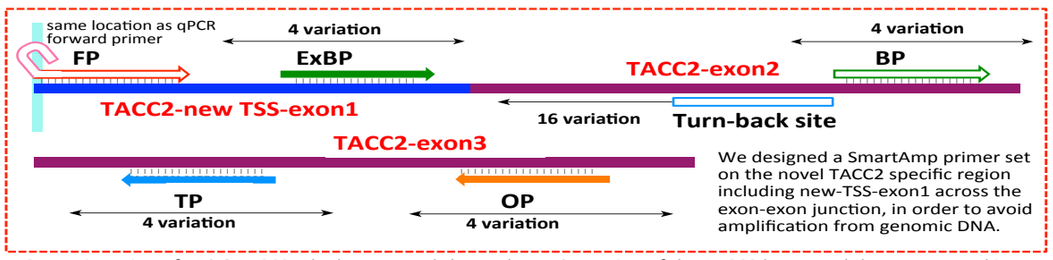


(3) We assessed the reaction based on RNA as the template in one step containing reverse transcription step.

Result2

Strategy for SmartAmp primer design



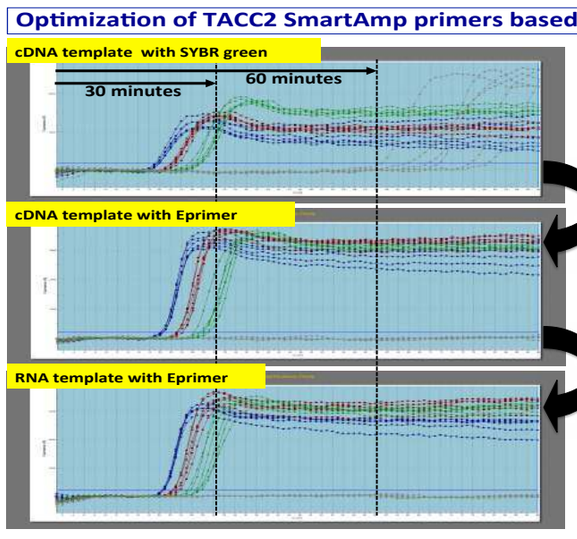


We designed a SmartAmp primer set on the novel TACC2 specific region including new-TSS-exon1 across the exon-exon junction, in order to avoid amplification from genomic DNA.

Genomic region of p10@TACC2. The lower panel shows the entire region of the TACC2 locus, and the upper panel is a zoomed view of p10@TACC2. Sky blue highlighted background indicates p10@TACC2 location in both panels.

In the best primer combination, the difference is big between CT values of template +/-. We validated all combination primer patterns and succeeded to develop a promising candidate of primer set to detect the novel TACC2 isoform by using SmartAmp.

Optimization of TACC2 SmartAmp primers based on novel TACC2 RNA

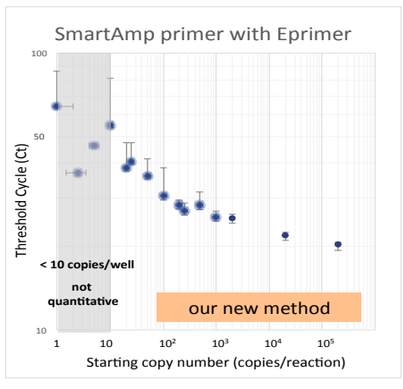


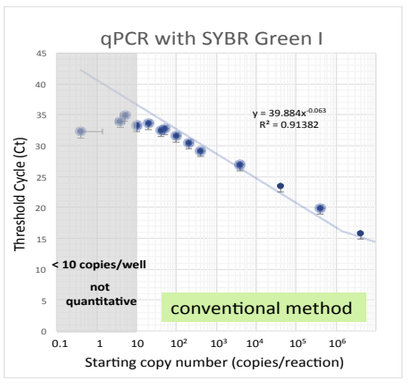
novel TACC2 cDNA or RNA 2.0×10^5, 2.0×10^4, 2.0×10^3 copies/μl

By changing detection fluorescence SYBR green into Eprimer, non-specific amplification of NTC disappeared.

We succeeded to the reaction based on RNA as the template in one step containing reverse transcription step.

Comparison of standard curve between SmartAmp and qPCR





We validated its amplification in low concentration RNA template and made standard curve. On the both curves, in low concentration (< 10 copies/well) the variation of value were large and not quantitative.

- We succeeded to develop a promising candidate of primer set to detect the novel TACC2 isoform by using SmartAmp.
- This candidate enable to quantify target RNA within 30 minutes.
- We have to improve the quantitativity of SmartAmp by increasing stating template volume etc.

Conclusion

✔ **It suggested that even when the sampling number is two, each average expression level indicates correct diagnosis with 99%CI.**

✔ **We succeeded to develop a promising candidate of primer set to detect the novel TACC2 isoform by using SmartAmp within 30 minutes.**

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