

# The differential gene expression of Notch receptors in primary and metastatic epithelial ovarian cancer sites

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## Summary

**Purpose of Investigation:** Notch signaling belongs among the candidate pathways for targeted therapies in ovarian cancer. While Notch 3 and Notch 1 overexpression has already been confirmed in ovarian carcinomas compared to their benign counterparts, the expression in metastatic sites is still not well known. The aim of the current study was to compare the relative expression of the four Notch gene receptors between primary and metastatic sites of ovarian carcinomas. **Materials and Methods:** Seventeen paired couples of tissues, from primary and metastatic sites of epithelial ovarian cancer, were collected during the cytoreductive surgery. For the study of the gene expression Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was used. The analysis of the results was performed with the Comparative C<sub>T</sub> Method ( $\Delta\Delta C_t$  Method). **Results:** Notch 3 and 2 were overexpressed compared to Notch 1 and 4 in both primary and metastatic sites. Notch 3 showed the highest differential expression, yet none of the four Notch genes reached statistical significance due to the differences between different samples. **Conclusions:** The lack of statistical difference in the expression of all four genes between primary and metastatic sites of epithelial ovarian cancer, potentially predicts same treatment response in all intrabdominal tumours and render Notch pathway a suitable and promising candidate for targeted therapies. The high heterogeneity in the expression between the samples, highlights even more the need of personalized therapies. Further studies should include more patients, examine the absolute expression, extend to all histological types, and focus on the Notch intracellular domain expression.

**Key words:** Notch; Receptors; Ovarian cancer; Expression; Metastasis.

## Introduction

Ovarian cancer is the most lethal malignancy of the female genital tract. The high mortality rate is mainly related to late diagnosis, as well as to chemotherapy resistance of the recurrent disease. Almost 61% of the cases are diagnosed in advanced stages as symptoms are non-specific [1]. Debulking surgery combined with adjuvant or neoadjuvant chemotherapy is the standard treatment approach. Complete cytoreduction is feasible in selected patients and no gross residual disease is associated with improved survival [2-4]. However, the exact effectiveness of the ultra-radical approach remains unclear and the need of randomized controlled trials is obvious [5]. Platinum- and taxane-based chemotherapy is effective in the majority of the cases [6, 7]. Unfortunately, most of them will relapse in the following years and the response rates of the following treatment lines are even lower [7, 8]. With the view of the need of new and more effective treatment options, new therapeutic agents and especially targeted therapies are studied [9].

In humans, Notch is a group of four (1-4) large single pass transmembrane glycoproteins (receptors). The related pathway is involved in multiple physiological cellular processes, while deviations of its normal function has been related to different diseases, including the development of syndromes and carcinogenesis [10]. Dysregulation of Notch receptors or ligands has been found in cervical, head and neck, renal, lung, breast, and other cancers [11].

In ovarian cancer, and especially in serous subtype, Notch belongs among the main pathways implicated in multiple cellular processes [12]. However, the study of the pathway began quite recently and the available data is still limited. In 2004, the first published data showed that Notch 3 is the main Notch receptor that is overexpressed at least three times in ovarian carcinomas compared to normal ovarian tissue [13, 14]. Immunohistochemistry and FISH (fluorescence *in situ* hybridization) studies found the presence of Notch 3 protein in the nucleus and the cytoplasm, as well as a direct relation between DNA copies and protein expression [15, 16]. Moreover, high levels of Notch 3

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mRNA and protein were found to be related to higher stage, lymph node and distal metastases, resistance to carboplatin, more aggressive phenotype, and poorer prognosis [17]. From a therapeutic point of view, the inhibition of the Notch 3 receptor with gamma secretase inhibitor (GSI) or small interfering RNA (siRNA) in cells that overexpress the receptor, caused reduced cellular proliferation and induced apoptosis, leading the authors to the conclusion that Notch 3 overexpression could be related to the pathogenesis of the high grade ovarian carcinomas [15]. Additionally, the combination of GSI with paclitaxel seems that increases the treatment response of the clinically carboplatin-resistant patients [18].

Notch 1 is the second best studied pathway after Notch 3. Hopfer *et al.* showed high to very high Notch 1 extracellular protein domain expression in OVCAR-3 and A2780 ovarian cancer cell lines [19]. However, in malignant cells isolated from tissue specimens, no difference in the expression of Notch 1 full length protein, as well as of the extracellular domain compared to that from borderline and benign tumours was found. The same study showed that Notch 1 mRNA was lower in carcinomas compared to adenomas [19]. Another study found that A2780 cells enriched with the Notch 1 NICD showed increased proliferation, leading to the conclusion that Notch 1 could potentially be involved in a few cellular procedures in ovarian cancer [19]. In 2010, Rose *et al.* found Notch 1 NICD expression in 76% of ovarian adenocarcinomas, as well as in OVCAR3, SKOV3, CaOV3 cell lines using Western Blot (WB)[20]. Inhibition of the pathways with siRNA led to high reduction of cellular proliferation [20], while the use of GSI before the treatment, showed that it increases the sensitivity to chemotherapy in cisplatin-resistant tumours [21]. In contrast to Hopfer *et al.*'s study, Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) experiments performed by Wang *et al.*, found increased levels of Notch 1 mRNA in ovarian cancer cells compared to normal ovarian cells, that corresponded to equally high level of protein in immunohistochemistry (IHC) and WB analysis [22].

Regarding Notch 2 and 4, the available data is extremely limited. Notch 2 NICD is not expressed in most serous, clear cell, and endometrioid ovarian carcinomas compared to the specimens from normal ovary. However, up to two-thirds of the mucinous carcinomas shows high levels of NICD protein [23]. Finally, in the only one available study for Notch 4, high expression was found in a cellular subgroup with cancer stem cells characteristics [24].

The aim of the current study was to analyse the expression of all four Notch mRNAs in both primary and metastatic (intra-abdominal) sites in order to clarify a potential therapeutic role of the inhibition of the Notch pathway.

Table 1. — *Characteristic of the patients enrolled in the study.*

No	17
Age (years)	
Range	40-73
Mean	57.29
FIGO Stage (%)	
IIIb	3 (17.65)
IIIc	13 (76.47)
IV	1 (5.88)
Histology (%)	
Serous	13 (76.47)
Endometrioid	3 (17.65)
Unknown adenoCa	1 (5.88)
Grade (%)	
2	2 (11.76)
3	14 (82.35)
Unknown	1 (5.88)

## Materials and Methods

Primary collected specimens from patients that underwent cytoreductive surgery in Theagenio Cancer Hospital (Thessaloniki, Greece) and Papageorgiou General Hospital (Thessaloniki, Greece) were used. Informed consent for the scientific use of biological material was obtained from the patients in accordance with the requirements of the ethics committees of the two hospitals. Patients of all ages, ethnic groups, and all histological types of ovarian / tubal / primary peritoneal carcinomas were included. Patients diagnosed with borderline ovarian tumors or with primary extra-ovarian malignancy (e.g. Krukenberg tumors) metastasized to the ovary were excluded. Moreover, patients having received any recent (< 2 years, or neoadjuvant) chemotherapy were also excluded, to decrease any possible effect of the treatment in the cellular/molecular pathways. Stage was at least IIIb in order to be collected sufficient tissue volume from the metastatic site. Paired couples of tissues, from primary and metastatic sites, were collected during the cytoreductive surgery. Metastatic specimens were mainly collected from the upper abdomen as an attempt was made to be pure metastasis, rather than extension of the primary tumor. Just after the collection, tissue underwent frozen section and were included into the study only if the malignancy was confirmed. Tissues were examined minutely by specialist pathologist to exclude the collection of unsuitable material (fat, necrotic or benign tissue) and stored in RNA later. Initially fifty pairs of specimens were collected and thirty of them met the inclusion criteria.

For the study of the four (1-4) Notch mRNA expression, qRT-PCR was selected. Total cellular mRNA was extracted using Trizol reagent. After the initial quality and quantity control of the extracted mRNA, only 17 out of 30 pairs (from primary and metastatic site) were suitable for qRT-PCR. Patients' characteristics are presented in Table 1. Extracted mRNA was used to create the complementary DNA (cDNA) using reverse transcriptase. The samples without genomic DNA contamination and characterized by proper amplification of the reverse transcription controls, giving specific PCR product for two different housekeeping genes [RARA (retinoic acid receptor a) and TP53], were further evaluated. A master mix kit was used for the qRT-PCR according to the manufacturer's instructions. The ABL (ABL proto-oncogene 1, non-receptor tyrosine kinase) was used as reference gene.

For the statistical analysis of the results the Comparative C<sub>T</sub>

Table 2. — Mean value of the relative expression ( $2^{-\Delta Ct}$ ) of the four Notch genes compared to the reference gene (ABL) in the primary ovarian site. The fold difference (FD) expresses the times difference of expression between two genes.  $P$  values  $\leq 0.05$  were considered as statistically significant.

Ovarian (primary)	Notch 1	Notch 2	Notch 3	Notch 4
Mean ( $2^{-\Delta Ct}$ )	0.296	1.024	1.697	0.481

Ovarian	FD	$p$ value
Notch 3 vs 1	5.73	0.006
Notch 3 vs 4	3.53	0.001
Notch 2 vs 1	3.46	<0.0001
Notch 2 vs 4	2.13	0.0062
Notch 3 vs 2	1.66	0.1183

Method (or  $\Delta\Delta Ct$  Method) was used. The values were further analysed by Excel and GraphPad Prism version 6. All data presented were the mean plus minus (+/-) standard error of the mean (S.E.M) of at least two independent experiments. All differences were confirmed as significant when  $p$ -values  $\leq 0.05$ , as evaluated by the two-tailed Student's  $t$ -test.

## Results

Table 2 and Figure 1 show the cumulative results of the qRT-PCR for the four Notch receptor genes. Based on the Comparative  $C_T$  Method that was used, the value of the  $2^{-\Delta Ct}$  represents the relative difference of the expression between the examined genes (Notch 1-4) and the reference gene (ABL). It is important to mention that the results of the present study, if examined separately in the primary and metastatic site, are difficult to be compared with findings of previous reports. This is due to the nature of the study, as the reported values represent relative expression of the examined genes, compared to the reference gene and not absolute values of comparison with benign or normal ovarian tissue.

In the primary sites of the ovarian cancer, Notch 1 showed the relative lower expression (mean  $2^{-\Delta Ct}$  = 0.296, range = 0.002-1.021) compared to the other three Notch genes, followed by Notch 4 that expressed the second lowest (mean = 0.481, range = 0.025-2.639). Notch 1 mRNA is expressed marginally higher ( $2^{-\Delta Ct}$  = 1.021) compared to ABL, in only one out of the 17 (5.88%) samples. For Notch 4, mRNA value was higher to that of ABL value in only 3/17 (17.6%) samples. On the other hand, Notch 2 and 3 were highly expressed (mean  $2^{-\Delta Ct}$  1.024 and 1.697 respectively) and the difference between their expression is not significant ( $p$  = 0.1183). Notch 3 was overexpressed compared to the reference gene in 8/17 (47%) of the samples. In two samples, the expression was significant higher ( $2^{-\Delta Ct}$  = 6.453 and 5.426) increasing the overall mean  $2^{-\Delta Ct}$

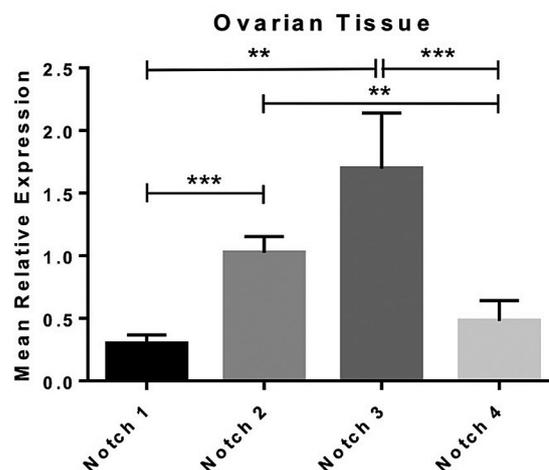


Figure 1. — Representation of the mean relative expression and standard errors of the mean (SEM) of the four Notch genes in the samples from the primary (ovarian) sites. Notch 2 and 3 are statistically significantly overexpressed compared to Notch 1 and 4.

value for this gene. Notch 3 expression was 5.73 and 3.53 times higher compared to Notch 1 and 4, respectively, and this was statistically significant ( $p$  values  $\leq 0.05$ ). Notch 2 mRNA was overexpressed compared to ABL in 9/17 (53%) of the samples. Contrary to Notch 3 (range = 0.034-6.453), in the case of Notch 2,  $2^{-\Delta Ct}$  values showed less variance ( $2^{-\Delta Ct}$  range = 0.196-2). Notch 2 was expressed 3.46 and 2.13 times higher to Notch 1 and 4, respectively, that is also statistically significant ( $p$  values  $\leq 0.05$ ).

Based on the most recent literature review (January 2017) this is one of the very few studies that examined the expression of the Notch receptors in metastatic ovarian specimens. The experimental results of the Notch gene expression from the metastatic sites are shown in Table 3 and Figure 2.

In metastatic sites, in accordance to the primary ones, Notch 1 and 4 showed the lowest gene expression. The mean  $2^{-\Delta Ct}$  of Notch 1 was 0.221 (range 0.004-1.110) while for Notch 4 it was 0.333 (range 0.019-2.462). In just only one sample (5.88%) the expression of these genes was higher to the reference gene.

On the other hand, Notch 2 showed the highest relative expression among the four genes (mean  $2^{-\Delta Ct}$  0.951, range 0.312-2.235). This was 4.3 and 2.86 times higher compared with Notch 1 and 4, respectively. Additionally, the mean  $2^{-\Delta Ct}$  of Notch 3 was 0.914 (range 0.119-2.828), corresponding to a 4.13 and 2.74-fold difference compared with Notch 1 and 4. For Notch 3 and 2, the expression of their gene was higher to the reference gene in 7 out of 17 (41.2%) samples, but in different samples for each gene. Thus, Notch 2 and 3 were highly expressed in the abdominal specimens, and their difference was statistically not

Table 3. — Mean relative expression of Notch 1-4 in the metastatic sites. *P* values  $\leq 0.05$  were considered as statistically significant.

Abdominal (metastatic)	Notch 1	Notch 2	Notch 3	Notch 4
Mean ( $2^{-\Delta\Delta Ct}$ )	0.221	0.951	0.914	0.333
Ovarian	FD			<i>p</i> value
Notch 3 vs 1	4.13			0.0031
Notch 3 vs 4	2.74			0.002
Notch 2 vs 1	4.3			0.0002
Notch 2 vs 4	2.86			0.0085
Notch 3 vs 2	0.96			0.864

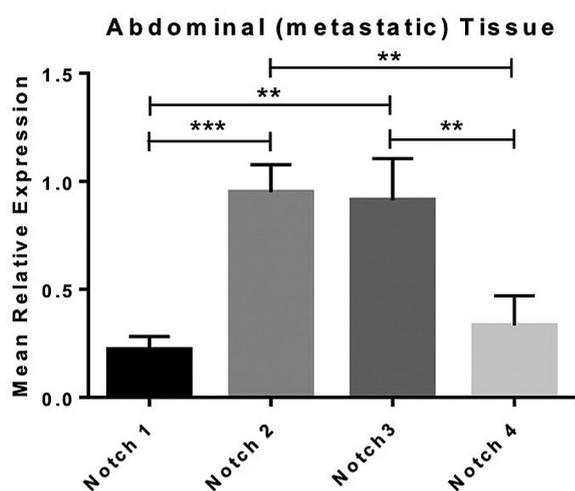


Figure 2. — Representation of the mean relative expression and standard errors of the mean (SEM) of the four Notch genes in the samples from the metastatic (abdominal) sites. Statistical significant over-expression of Notch 2 and 3, compared to Notch 1 and 4 is noted.

significant ( $p = 0.864$ ). The higher, even borderline and statistically not significant, expression of Notch 2 compared to Notch 3 is quite interesting and will be discussed further in the next chapters. Additionally, worthy of notice that regardless the overall high expression of Notch 2 and Notch 3 also in the metastatic specimens, the values of  $2^{-\Delta\Delta Ct}$  (relative expression) of isolated samples, as well as the range of the values, were lower compared to these of the samples from the ovarian tissues. In other words, the fluctuation of the relative expression was more homogeneous in the abdominal samples.

The mean relative expression of all four genes were higher in the ovarian samples compared to the metastatic sites. The difference was higher for Notch 3 (1.857x) and lower for Notch 2 (1.077x) as this gene was highly expressed also in metastatic sites. These differences for Notch

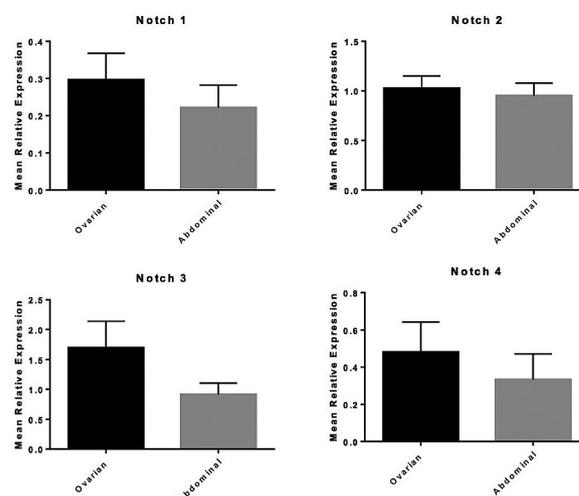


Figure 3. — Comparison of the mean relative expression and the standards errors of the mean of the four Notch genes, between ovarian and abdominal specimens. The expression in the samples from the primary sites are higher compared to the metastatic, yet none of the differences reaches statistical significance.

1 and 4 gained intermediate values (1.338x and 1.445x respectively). Moreover, and most importantly, none of the above differences (including the Notch 3 that showed the highest one) reached statistical significance, as all *t*-tests  $> 0.05$  (Notch 1 = 0.373, Notch 2 = 0.643, Notch 3 = 0.097, and Notch 4 = 0.407) (Figure 3).

The values of the  $2^{-\Delta\Delta Ct}$  algorithm expressed the difference of the relative expression of each of the four Notch genes in comparison to the ABL, between primary and metastatic site. Using this algorithm, we can identify the isolated samples that are differentially expressed in the metastatic sites and quantify the difference (data not shown). Thus, 9/17 (52.94%) of the samples expressed Notch 1 gene higher in the metastatic specimens ( $2^{-\Delta\Delta Ct}$  range = 0.007-0.859), while the other eight samples in the primary (ovarian) specimens ( $2^{-\Delta\Delta Ct}$  range = 1.301-88.647). In the case of Notch 2, in 8/17 (47.06%) of the samples the gene was expressed higher in the metastatic specimens ( $2^{-\Delta\Delta Ct}$  range = 0.337-0.796) and in 9/17 in the primary specimens ( $2^{-\Delta\Delta Ct}$  range = 1.165-3.531). Additionally, the majority (11/17) of the samples expressed Notch 3 higher in the ovarian specimens ( $2^{-\Delta\Delta Ct}$  range = 1.292-18.765), as only 6/17 (35.29%) of the samples expressed it higher in the abdominal tumours ( $2^{-\Delta\Delta Ct}$  range = 0.08-0.655). Finally, 7/17 (41.18%) of the specimens expressed Notch 4 higher in the metastases ( $2^{-\Delta\Delta Ct}$  range = 0.079-0.785), while the remaining ten specimens in the primary (ovarian) sites ( $2^{-\Delta\Delta Ct}$  range = 1.516-10.196).

## Discussion

Targeted therapies belong to the most challenging treatment approaches of epithelial ovarian cancer. The resistance to the conventional chemotherapeutic agents after the initial usually quite good response, render necessary the study of the multiple signalling pathways involved in the pathogenesis, progression, metastasis, and recurrence of the disease. On the other hand, it is known that each tumour includes multiple cellular clones with different malignant potential and therefore theoretical different response in therapies, especially targeted. In the case of the epithelial ovarian carcinomas with the typical intraperitoneal dissemination, the study of a pathway should involve not only samples from the primary (ovarian) site, but also from distal intra-abdominal metastatic sites, especially from the upper abdomen. The hypothesis is that a targeted therapy would be effective only if the targeted pathway/receptor is expressed at the same (high) level in all tumour masses.

This study is one of the very few studies that used snap frozen material directly from the patients. Moreover, all four Notch genes were examined and this is one of the first sets of experiments that not only used tissues from metastatic sites, but also compared the results with that from the primary sites. Additionally, an attempt was made to extract RNA with a high quality (pathologist involvement, low threshold for exclusion of low quality material, multiple quantity and quality controls, etc.). Worthy of notice is that this study examined the relative expression of the Notch genes in comparison with the reference gene and not the absolute in comparison with normal tissue or benign tumours.

In the present study, a low expression of the Notch 1 gene in the samples from the primary sites compared to the reference gene (ABL) was found. Previous findings were controversial. However, as noticed before, these findings cannot directly be compared to these of the previous studies, as here the authors used relative and not absolute Notch genes expression. Thus, this lower expression of the Notch 1 gene could only show a tendency and not definite lower absolute expression.

Based on the same limitations, the authors found low levels of the Notch 4 mRNA in the primary sites. The lack of almost any previous finding regarding this gene make the present results interesting but, on the other hand, unsuitable for any type of comparison.

Notch 2 showed one of the most interesting findings of the present study. For the very first time, a high expression, almost equivalent to the Notch 3 (that is known to be overexpressed in ovarian cancer) was found. Unfortunately, the collected sample of mucinous tumour, that theoretically expresses higher the gene, was not suitable for further evaluation. The high expression of Notch 2 mRNA is in contrast to previous findings of Galic *et al.* that showed low level of the NICD [23]. There are multiple possible explanations

for this discrepancy; firstly, the current study evaluates relative expression of the genes. Even if it is quite possible that the absolute expression of Notch 2 could be high as well (9/17 samples overexpress the gene), this cannot be definitely confirmed in this study. Moreover, the number of the samples are not directly comparable due to the different nature of the two studies (prospective vs retrospective). Another reason could be the different study populations. Finally, and quite likely, there is a chance that the transcribed mRNA is not translated into the protein due to multiple reasons (e.g. unstable mRNA).

The known overexpression of Notch 3 gene in ovarian carcinomas is confirmed in the present study. It is important to notice that these high levels are mainly determined by the high to very high expression of isolated samples (47% of the specimens), rather than by an overall overexpression of all the samples. This is in accordance to the study of Jung *et al.* that showed overexpression of Notch 3 mRNA in about 63% of serous carcinomas compared to benign ovarian tumours[25].

Regarding the metastatic (abdominal) specimens, Notch 2 and 3 are the main genes highly expressed in the intra-abdominal spread tumors, while the expression of Notch 1 and Notch 4 is low, in accordance to the levels of these genes in the primary sites. The high relative expression of Notch 2 in combination with the respectively high expression in the ovarian samples seems interesting and promising.

The comparison of the expression between primary and metastatic sites is the main aim of the study. The authors notice that all four Notch genes were expressed higher in the ovarian samples. Among the genes, Notch 3 showed the highest difference. Studying the collected specimens sample by sample, the authors found a very high diversity in the expression that varied from under-expression to over-expression in the primary or in the metastatic site. This high variety was also found in previous studies and led the present authors to hypothesize that is a characteristic of the studies or of these particular genes [25]. However, the overall difference in the relative expression did not reach statistical difference for none of the four genes, mainly due to the high heterogeneity of the expression between the samples.

## Conclusion

In conclusion, herein the authors found a high relative expression of Notch 2 and 3 mRNA and low of Notch 1 and 4 in both the primary and metastatic sites of epithelial ovarian carcinomas. All four genes were highly expressed in the ovarian samples compared to the abdominal ones, even if none of the differences reached statistical significance. More importantly, the aforementioned direct relation between Notch DNA and protein, as previous studies found, leads the present authors to believe that their find-

ings could potentially be confirmed at the protein level as well. From a clinical point of view, the equivalent expression in all intra-abdominal and pelvic samples, render Notch signalling pathway a candidate for targeted therapies. Notch 1 and 3 should probably be the first targets; however, this study revealed an interesting possible role of Notch 2 as well. Nevertheless, the high heterogeneity in the expression further highlights the need of personalised therapies. A possible clinical scenario could be the below; histological specimens from the primary debulking or guided biopsy are examined for Notch 3 expression. Patients with high expression of Notch 3 could be considered platinum-resistant and suitable for Notch targeted treatment with siRNA or GSI (neoadjuvant or adjuvant). Alternatively, Notch pathway could be the target in case of recurrent disease (2<sup>nd</sup> or 3<sup>rd</sup> line) as theoretically should be effective in all intrabdominal tumours, like this study indirectly showed.

Finally, future studies should overcome the current limitations. Thus, more patients should be recruited and the specimens' collection should include all ovarian cancer histological types. Ideally, the study of the absolute expression and especially the protein expression (NICD) would clarify more the exact role of Notch pathway in ovarian carcinomas and drive future clinical trials.

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