NOTCH2 expression is decreased in epithelial ovarian cancer and is related to the tumor histological subtype

Vijaya Galic^{1*}, Carrie J. Shawber¹, Claire Reeves¹, Monjri Shah², Aino Murtomaki¹, Jason Wright¹, Thomas Herzog¹, Guo Xia Tong³ and Jan Kitajewski¹

*Correspondence: vlg2110@columbia.edu

¹Department of Obstetrics and Gynecology, Columbia University Medical Center, New York, New York, United States of America.

²Department of Obstetrics and Gynecology, University of Alabama, Birmingham Alabama, United States of America.

³Department of Pathology, Columbia University Medical Center, New York, New York, United States of America.

Abstract

Background: Notch family members function as both oncogenes and tumor suppressors. *NOTCH2* is down-regulated in colon cancer, and reduced expression is associated with a less differentiated, more aggressive phenotype, and reduced overall survival. *NOTCH2* has also been shown to have pro-apoptotic and growth suppressive effects in thyroid carcinoma, and carcinoid tumors. The expression pattern of *NOTCH2* in ovarian cancer is unknown.

Methods: An immunohistochemical analysis using a polyclonal antibody to the *NOTCH2* intracellular domain was performed on a total of 119 ovarian carcinomas, and 7 serous borderline tumors, arranged onto tissue arrays. Normal ovarian and fallopian tube epithelium were used as controls. Specimens were scored as low or high *NOTCH2* expression. The score distributions for the subtypes were analyzed with the chi square test.

Results: Fifty two of 61 (85.2%) papillary serous, eight of 13 (61.5%) clear cell, and 23 of 30 (76.7%) endometrioid, demonstrated negative or lower *NOTCH2* expression than normal fallopian tubal epithelium or ovarian surface epithelium. In contrast, 10 of 15 (66.7%) mucinous carcinomas had a high level of *NOTCH2* expression and consistently demonstrated intense polarized staining ($P < .001$). The apical expression of *NOTCH2* protein present in the normal fallopian tube epithelium and many borderline tumors was absent in the high grade carcinomas, most notably in papillary serous.

Conclusion: Decreased *NOTCH2* expression is associated with the poorly differentiated serous epithelial ovarian carcinoma histology. Further studies are needed to assess the functional role of *NOTCH2* in ovarian cancer and its effect on prognosis.

Keywords: *NOTCH2*, ovarian cancer, tumor suppressor, immunohistochemistry

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy. In 2012, there were an estimated 22,280 women diagnosed with ovarian cancer with 15,500 deaths [1]. The five-year overall survival is approximately 45%. Ovarian cancer collectively encompasses multiple histological types. Of these, the papillary serous histology accounts for about 60% [2]. Less common are mucinous, clear cell, and endometrioid tumors [2]. Each histological type is distinct with respect to its pathogenesis and clinical behavior, including response to chemotherapy [3]. Arguably the most important prognostic factor in ovarian cancer is stage. It is plausible that ovarian cancer, like colon or cervical cancer arises from a precursor lesion. Mucinous carcinomas and low grade serous carcinomas have been shown to co-exist with borderline tumors of the corresponding histology [4,5]. Recently, clear cell carcinomas have been shown to arise from areas of atypical endometriosis with loss of expression of *ARID1A* [6]. A clear precursor lesion has not been identified for papillary serous carcinomas, which tend to present at an advanced stage. A subset of presumed serous carcinomas of

the ovary or fallopian tube, however, may actually arise from intraepithelial carcinomas of the distal fallopian tube [7]. Lack of identification of a papillary serous precursor has precluded the development of effective screening tests and hampered the elucidation of early carcinogenic events in epithelial ovarian carcinoma.

Several regulators of cell fate, most notably *P53* [8], *BRCA1/2* [9], *PTEN*, and members of the *PIK3* pathway [10] have been investigated in the context of ovarian cancer. Recently identified regulators of cell fate include members of the *NOTCH* signaling pathway. This signaling pathway is an evolutionarily conserved intercellular communication mechanism, which is mediated by the interaction of receptors and the cognate ligands on the cell membrane of neighboring cells. There are four *NOTCH* receptors (*NOTCH* 1-4) and five canonical ligands (Delta-like 1, 3, and 4 and Jagged-1 and -2) identified in mammals. *NOTCH* signaling is involved in many cellular processes including cell differentiation, proliferation, survival, and apoptosis. *NOTCH* signaling contributes to the specification of cell lineages, tissue patterning and morphogenesis, stem cell maintenance, and

Figure 1. *NOTCH2* expression in the normal epithelium. **A.** Upper panel H&E staining of normal fallopian tube epithelium. Lower panel *NOTCH2* staining (green) present at the apical and basal areas of the cell membrane. Nuclei are blue. **B.** Upper panel H&E staining of normal ovarian surface epithelium. Lower panel *NOTCH2* staining (green) present at the apical and basal areas of the cell membrane. Nuclei are blue. 20X magnification.

tissue homeostasis during embryonic development or in adult life [11]. Abnormal *NOTCH* signaling is associated with various human cancers. Though classically studied in the context of tumor angiogenesis [12], *NOTCH* family members have also been implicated in tumor cell growth and proliferation as well as in the epithelial to mesenchymal transition [13,14]. *NOTCH* family members function in a cell type-specific and cell context-dependent manner and may function as both oncogenes as well as tumor suppressors. *NOTCH1*, 2 and 3, have been implicated in tumor growth regulation with the type of effect being cancer specific. *NOTCH1* overexpression has been shown to promote tumor cell proliferation and invasion in breast cancer [15] and proliferation in ovarian cancer cell lines [16,17]. In colon cancer, *NOTCH1* expression has been shown to be an adverse prognostic indicator [18]. *NOTCH3* likewise has also been identified as an oncogene in serous ovarian cancer, which frequently overexpresses *NOTCH3* as a result of gene amplification [19-21]. *NOTCH3* overexpression in serous ovarian carcinomas has been correlated with carboplatin resistance [22]. In contrast to *NOTCH1* and *NOTCH3*, *NOTCH2* has been shown to play a tumor suppressive role in many cancers including colon, medullary thyroid [23], carcinoid [24], and colon cancer [18,25] and may correlate with increased differentiation in both colon [25] and breast cancer [26]. The role of *NOTCH2* has not been studied in the setting of ovarian cancer. We report here our findings of *NOTCH2* expression in normal ovary, fallopian tube, serous borderline tumors of the

ovary and ovarian carcinomas.

Materials and methods

Archival pathology specimens were utilized in this study. Tissues were collected from surgical specimens following primary cytoreductive surgery for fallopian tube, primary peritoneal or ovarian cancer, or following surgery for benign gynecologic indications. Specimens were formalin fixed and paraffin embedded at the time of surgery. Tissue microarrays (TMA) containing 61 papillary serous carcinomas (PS), 13 clear cell carcinomas (CC) 30 endometrioid carcinomas (EC), and 15 mucinous carcinomas (MC) (119 cases evaluated) were assembled by three cores (0.6 mm) of tumor from a representative tissue block of each case. All H&E slides were reviewed and the pathologic diagnoses were confirmed. In addition, routine tissue sections of nine serous borderline tumors were also included. The uninvolved ovarian surface epithelium and fallopian tubal epithelium served as normal tissue controls.

Immunohistochemistry of *NOTCH2* with a rabbit polyclonal antibody to *NOTCH2* (Abcam ab8926 1:100 dilution, Abcam, Cambridge, MA) was performed on the 4 micron sections from either TMA or regular tissue blocks as suggested by the manufacturer. This antibody had been previously validated by the manufacturer. Additionally, validation of the antibody was performed within our laboratory. The staining pattern was identical to that of a *NOTCH2* antibody from a different manufacturer, was similar to what has previously been described by Zhang et al. in the mouse ovary, and produced a staining pattern distinct from the staining patterns of *NOTCH1* and *NOTCH3* (Supplement Figure S1). Immunohistochemistry of *NOTCH2* with a rabbit polyclonal antibody (1:100 dilution, Abcam, Cambridge, MA) was performed on the 4 micron sections from either TMA or regular tissue blocks as suggested by the manufacturer. In brief, the sections were deparaffinized, rehydrated in decreasing concentrations of ethanol, and subjected to antigen retrieval in Target Retrieval Buffer (Dako, Carpinteria, CA) for 30 minutes. The sections were blocked in 3% BSA and incubated with the *NOTCH2* antibody for 10 hours at 4°C. After washing with PBS, the sections were incubated with a fluorescent conjugated secondary antibody, Alexa Fluor 488 (Invitrogen), at a dilution of 1:10,000 for 30 min at 25°C. The slides were coverslipped with Vectashield mounting medium containing DAPI for visualization of the nuclei (Vector laboratories, Burlingame, CA). The staining was examined and photographed using a Nikon fluorescent microscopy system with a digital camera. The staining was scored with a two tier system (high or low) independently by three observers based on the intensity and extensiveness of staining. Consensus was reached by re-evaluation at conference on cases with initial disagreement.

The data of staining intensities were analyzed using the chi square test included in the Microsoft excel statistical package.

Figure 2. NOTCH2 staining in serous borderline tumors. Upper panels H&E staining of two serous borderline specimens. Lower panels NOTCH2 staining of corresponding specimens (green). Nuclei are blue. A. specimen demonstrating polarized NOTCH2 staining. B. specimen demonstrating diffuse cytoplasmic NOTCH2 staining. Nuclei are blue. 20X magnification.

Figure 3. A. Upper panel H&E high grade papillary serous ovarian carcinoma. Lower panel NOTCH2 staining (green) is absent. Nuclei are blue. B. Upper panel H&E staining of mucinous ovarian carcinoma. Lower panel NOTCH2 staining (green) demonstrating high level of NOTCH2 expression with intense polarized staining. Nuclei are blue. 20X magnification.

A p value of <.05 was considered statistically significant.

Results

As shown in **Figure 1A** and **1B**, NOTCH2 staining was detected

Table 1. NOTCH2 Expression and Histology.

NOTCH2	Papillary Serous	Clear Cell	Endometrioid	Mucinous
Low	52	8	23	5
High	9	5	7	10
Totals	61	13	30	15

in the normal fallopian tubal epithelium and ovarian surface epithelium. The staining was strong and mainly present at the apical membranes and to a lesser extent was also seen at the basal areas of these epithelial cells. The smooth muscle cells of the fallopian tube and ovarian sex cord-stromal cells were consistently negative for NOTCH2. Four of seven of the serous borderline tumors in this study demonstrated apical distribution of NOTCH2 in the neoplastic cells as seen in the normal fallopian tube and the ovarian surface epithelium; however basal distribution of NOTCH2 was decreased in these cells. A representative specimen is shown in **Figure 2A**. In the remaining three cases of borderline serous adenoma, the neoplastic cells had intense diffuse cytoplasmic staining of NOTCH2 as seen in the representative specimen in **Figure 2B**. For the malignant epithelial tumors of the ovary, 52 of 61 (85.2%) PS, eight of 13 (61.5%) CC, and 23 of 30 (76.7%) EC, demonstrated negative or lower NOTCH2 expression than normal fallopian tubal epithelium or ovarian surface epithelium tumors. **Figure 3A** is a representative PS with negative expression. In contrast, 10 of 15 (66.7%) MC had a high level of NOTCH2 expression and consistently demonstrated intense polarized staining as shown in **Figure 3B** (P <.001) (**Table 1**).

Discussion

In this study, we investigated the expression of NOTCH2 in normal ovarian surface epithelium, fallopian tubal epithelium, and various histologic types of ovarian carcinomas utilizing immunohistochemistry. We have demonstrated that NOTCH2 is cell membrane-associated and expressed both in the apical and basal sides of the cell in the normal ovarian surface epithelium and fallopian tubal epithelium. Epithelial cells demonstrate polarized expression of several other molecules including those integral for the formation of tight junctions [27]. Further studies are needed to determine whether NOTCH2 is related to the formation and maintenance of epithelial tight junctions on ovarian epithelial cells. Of note, many of the serous borderline tumors studied contained areas in which the polarity of NOTCH2 expression was preserved. Even in those tumors with non-polarized membranous staining, the overall staining intensity was very high. It is not possible from immunohistochemistry observations alone to establish whether the association between high NOTCH2 expression and a non-invasive phenotype will translate into a causal relationship between loss of NOTCH2 and invasion. Further functional studies are needed to explore this.

NOTCH2 expression was present in only a small percentage of the papillary serous ovarian cancer specimens. In the context

of the tumor suppressive role of *NOTCH2* in colon cancer cells, it is plausible that the poorly differentiated serous carcinomas are also down-regulating *NOTCH2*. Further studies are needed to clarify whether *NOTCH2* down-regulation is an early causal event in high grade serous carcinoma carcinogenesis, or if the present observation is simply coincidental with a less differentiated state. While the endometrioid tumors, and clear cell specimen sets also had more tumors with low *NOTCH2* expression than high *NOTCH2* expression, the difference was not as pronounced as in the serous histology tumors. While the small number of specimens in these sets limits the conclusions which may be drawn, it is plausible that given the distinct carcinogenic pathways by which these tumors arise, *NOTCH2* down-regulation may play a less important role in non-serous carcinomas.

Down-regulation of *NOTCH2* has also been documented in colon carcinoma. Loss of expression was more commonly associated with poorly differentiated tumors [25]. Poorly differentiated tumor cells lacking *NOTCH2* expression regain it after forced differentiation by butyrate exposure [25]. Interestingly, *NOTCH2* expression was preserved in the majority of the ovarian mucinous tumors in the present study. This is consistent with the clinical behavior of these tumors, which unlike serous tumors, are more commonly low grade and confined to the ovary at the time of diagnosis [2].

One limitation of this study is that while the *NOTCH2* antibody used in this study recognizes the intracellular domain, it does not specifically identify *NOTCH2* involved in active signaling. In the case of *NOTCH3*, however, the expression level of the intracellular domain alone has been shown to correlate with clinical behavior [20,22]. Another limitation of this study is that while expression differences were noted, and expression was lower in poorly differentiated tumors, no direct conclusions may be drawn regarding whether *NOTCH2* is affecting tumor behavior. Additionally, it is unclear whether loss of *NOTCH2* expression simply coincides with a poorly differentiated state.

The present study explores the pattern of *NOTCH2* expression in epithelial ovarian cancer. As has been previously demonstrated in colon cancers, *NOTCH2* expression was lost in the vast majority of poorly differentiated tumors. It is plausible therefore that *NOTCH2* is related to tumor suppression in ovarian cancer as it is in colon cancer. Further functional experiments are required to validate the observations of the present study and to determine the role of *NOTCH2* signaling in ovarian cancer cells.

Additional files

Supplement Figure S1

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Galic was responsible for study design, performed the

immunohistochemistry on the tissue array, scored the array, interpreted the findings and prepared the manuscript. Shawber was responsible for the validation of the antibody for immunohistochemistry. Additionally she scored the array immunohistochemistry, provided guidance for photography and assisted with interpretation of the results. Tong constructed the tissue array, scored the immunohistochemistry and assisted with interpretation of the results and development of the manuscript. Reeves and Murtomaki assisted with photography of the array, interpretation of the results and preparation of the figures and manuscript. Herzog and Wright supplied surgical specimens and assisted with interpretation of the results and development of the manuscript. Kitajewski provided mentorship and guidance for the project overall and oversaw all aspects of study and final manuscript.

Acknowledgement

This work was supported by a grant from the NIH 5R01CA126673 to J.K. and C.S.

Publication history

Editor: Bin Guan, Johns Hopkins Medical Institutions, USA.

EIC: Markus H. Frank, Harvard Medical School, USA.

Received: 29-May-2013 Revised: 31-Jul-2013

Accepted: 08-Aug-2013 Published: 16-Aug-2013

References

1. American Cancer Society. **Cancer Facts & Figures 2012**. Atlanta: American Cancer Society; 2012. | [Pdf](#)
2. Schiavone MB, Herzog TJ, Lewin SN, Deutsch I, Sun X, Burke WM and Wright JD. **Natural history and outcome of mucinous carcinoma of the ovary**. *Am J Obstet Gynecol*. 2011; **205**:480 e1-8. | [Article](#) | [PubMed](#)
3. Winter WE, 3rd, Maxwell GL, Tian C, Carlson JW, Ozols RF, Rose PG, Markman M, Armstrong DK, Muggia F and McGuire WP. **Prognostic factors for stage III epithelial ovarian cancer: a Gynecologic Oncology Group Study**. *J Clin Oncol*. 2007; **25**:3621-7. | [Article](#) | [PubMed](#)
4. Malpica A, Deavers MT, Lu K, Bodurka DC, Atkinson EN, Gershenson DM and Silva EG. **Grading ovarian serous carcinoma using a two-tier system**. *Am J Surg Pathol*. 2004; **28**:496-504. | [Article](#) | [PubMed](#)
5. Hart WR. **Mucinous tumors of the ovary: a review**. *Int J Gynecol Pathol*. 2005; **24**:4-25. | [Article](#) | [PubMed](#)
6. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConchey MK, Anglesio MS, Kalloger SE, Yang W et al. **ARID1A mutations in endometriosis-associated ovarian carcinomas**. *N Engl J Med*. 2010; **363**:1532-43. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
7. Chivukula M, Niemeier LA, Edwards R, Nikiforova M, Mantha G, McManus K and Carter G. **Carcinomas of Distal Fallopian Tube and Their Association with Tubal Intraepithelial Carcinoma: Do They Share a Common "Precursor" Lesion? Loss of Heterozygosity and Immunohistochemical Analysis Using PAX 2, WT-1, and P53 Markers**. *ISRN Obstet Gynecol*. 2011; **2011**:858647. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
8. Kohler MF, Kerns BJ, Humphrey PA, Marks JR, Bast RC, Jr. and Berchuck A. **Mutation and overexpression of p53 in early-stage epithelial ovarian cancer**. *Obstet Gynecol*. 1993; **81**:643-50. | [PubMed](#)
9. Pal T, Permuth-Wey J, Betts JA, Krischer JP, Fiorica J, Arango H, LaPolla J, Hoffman M, Martino MA, Wakeley K, Wilbanks G, Nicosia S, Cantor A and Sutphen R. **BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases**. *Cancer*. 2005; **104**:2807-16. | [Article](#) | [PubMed](#)
10. Willner J, Wurz K, Allison KH, Galic V, Garcia RL, Goff BA and Swisher EM. **Alternate molecular genetic pathways in ovarian carcinomas of common histological types**. *Hum Pathol*. 2007; **38**:607-13. | [Article](#) | [PubMed](#)
11. Wang MM. **Notch signaling and Notch signaling modifiers**. *Int J Biochem*

- Cell Biol.* 2011; **43**:1550-62. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
12. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetsee S, Boland P, Gale NW, Lin HC, Yancopoulos GD and Thurston G. **Blockade of DLL4 inhibits tumour growth by promoting non-productive angiogenesis.** *Nature.* 2006; **444**:1032-7. | [Article](#) | [PubMed](#)
 13. Bao B, Wang Z, Ali S, Kong D, Li Y, Ahmad A, Banerjee S, Azmi AS, Miele L and Sarkar FH. **Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells.** *Cancer Lett.* 2011; **307**:26-36. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 14. Yang Y, Ahn YH, Gibbons DL, Zang Y, Lin W, Thilaganathan N, Alvarez CA, Moreira DC, Creighton CJ, Gregory PA, Goodall GJ and Kurie JM. **The Notch ligand Jagged2 promotes lung adenocarcinoma metastasis through a miR-200-dependent pathway in mice.** *J Clin Invest.* 2011; **121**:1373-85. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 15. Wang J, Fu L, Gu F and Ma Y. **Notch1 is involved in migration and invasion of human breast cancer cells.** *Oncol Rep.* 2011; **26**:1295-303. | [Article](#) | [PubMed](#)
 16. Rose SL, Kunnimalaiyaan M, Drenzek J and Seiler N. **Notch 1 signaling is active in ovarian cancer.** *Gynecol Oncol.* 2010; **117**:130-3. | [Article](#) | [PubMed](#)
 17. Wang M, Wu L, Wang L and Xin X. **Down-regulation of Notch1 by gamma-secretase inhibition contributes to cell growth inhibition and apoptosis in ovarian cancer cells A2780.** *Biochem Biophys Res Commun.* 2010; **393**:144-9. | [Article](#) | [PubMed](#)
 18. Chu D, Zhang Z, Zhou Y, Wang W, Li Y, Zhang H, Dong G, Zhao Q and Ji G. **Notch1 and Notch2 have opposite prognostic effects on patients with colorectal cancer.** *Ann Oncol.* 2011; **22**:2440-7. | [Article](#) | [PubMed](#)
 19. Park JT, Li M, Nakayama K, Mao TL, Davidson B, Zhang Z, Kurman RJ, Eberhart CG, Shih Ie M and Wang TL. **Notch3 gene amplification in ovarian cancer.** *Cancer Res.* 2006; **66**:6312-8. | [Article](#) | [PubMed](#)
 20. Jung SG, Kwon YD, Song JA, Back MJ, Lee SY, Lee C, Hwang YY and An HJ. **Prognostic significance of Notch 3 gene expression in ovarian serous carcinoma.** *Cancer Sci.* 2010; **101**:1977-83. | [Article](#) | [PubMed](#)
 21. Cancer Genome Atlas Research Network. **Integrated genomic analyses of ovarian carcinoma.** *Nature.* 2011; **474**:609-15. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 22. Park JT, Chen X, Trope CG, Davidson B, Shih Ie M and Wang TL. **Notch3 overexpression is related to the recurrence of ovarian cancer and confers resistance to carboplatin.** *Am J Pathol.* 2010; **177**:1087-94. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 23. Truong M, Cook MR, Pinchot SN, Kunnimalaiyaan M and Chen H. **Resveratrol induces Notch2-mediated apoptosis and suppression of neuroendocrine markers in medullary thyroid cancer.** *Ann Surg Oncol.* 2011; **18**:1506-11. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 24. Pinchot SN, Jaskula-Sztul R, Ning L, Peters NR, Cook MR, Kunnimalaiyaan M and Chen H. **Identification and validation of Notch pathway activating compounds through a novel high-throughput screening method.** *Cancer.* 2011; **117**:1386-98. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 25. Chu D, Zheng J, Wang W, Zhao Q, Li Y, Li J, Xie H, Zhang H, Dong G, Xu C, Li M, Chen D and Ji G. **Notch2 expression is decreased in colorectal cancer and related to tumor differentiation status.** *Ann Surg Oncol.* 2009; **16**:3259-66. | [Article](#) | [PubMed](#)
 26. Fu YP, Edvardsen H, Kaushiva A, Arhancet JP, Howe TM, Kohaar I, Porter-Gill P, Shah A, Landmark-Hoyvik H, Fossa SD, Amb S, Naume B, Borresen-Dale AL, Kristensen VN and Prokunina-Olsson L. **NOTCH2 in breast cancer: association of SNP rs11249433 with gene expression in ER-positive breast tumors without TP53 mutations.** *Mol Cancer.* 2010; **9**:113. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 27. Zhu Y, Maric J, Nilsson M, Brannstrom M, Janson PO and Sundfeldt K. **Formation and barrier function of tight junctions in human ovarian surface epithelium.** *Biol Reprod.* 2004; **71**:53-9. | [Article](#) | [PubMed](#)

Citation:

Galic V, Shawber CJ, Reeves C, Shah M, Murtomaki A, Wright J, Herzog T, Tong GX and Kitajewski J. **NOTCH2 expression is decreased in epithelial ovarian cancer and is related to the tumor histological subtype.** *Pathol Discov.* 2013; **1**:4. <http://dx.doi.org/10.7243/2052-7896-1-4>