

# Tissue Microarrays for Testing Molecular Biomarkers of Cervical Intraepithelial Neoplasia

## Feasibility Study

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### Objective

To test the possibility of creating tissue microarrays of premalignant lesions of the cervix.

### Study Design

Paraffin-embedded blocks of 240 cervical tissue specimens were sampled. Lesions from benign squamous and glandular epithelium through various grades of cervical intraepithelial neoplasia (CIN) to frank carcinoma of squamous and glandular origin were cored with a 0.6-mm needle and arrayed in 4 tissue blocks. Sections of these blocks were stained with hematoxylin-eosin (H-E) and evaluated as to adequacy of tissue cores, representativity of the material and correspondence to the original diagnosis. Immunohistochemical staining with p16 and a novel marker C4.8(4/2/#1) was performed.

### Results

In >80% of cases sufficient material from the lesion could be obtained. No or inadequate material was seen in 6% of cases. The core sample did not correspond to the original diagnosis in 12% of cases. The reason was mainly a discrepancy in the grade of the CIN. Discrepancies in diagnoses oc-

curred in only premalignant lesions. Immunohistochemical staining could reliably be performed and evaluated on all tissue cores.

### Conclusion

Tissue microarrays of cervical intraepithelial lesions are technically feasible and can be created reliably. The key to success is a careful and repeated comparison of the tissue block with the corresponding H-E section. Tissue microarrays of preinvasive cervical lesions may allow high throughput analysis of emerging molecular biomarkers in cervical carcinogenesis. (Acta Cytol 2005;49:0000-0000)

**Tissue microarrays have the potential to vastly facilitate the testing of emerging biomarkers of cervical carcinogenesis because numerous samples in a single paraffin block will produce large data in a standardized fashion.**

**Keywords:** cervical intraepithelial neoplasia, mass screening, tissue microarrays.

The application of molecular techniques to the study of cervical carcinoma and its precursors has led to rapidly developing new insights into the mechanisms of cervical carcinogenesis.<sup>1,2</sup> Specific biomarkers associated with the progression from normal epithelium through the various grades of intraepithelial neoplasia to frank carcinoma have been recognized.<sup>3-5</sup>

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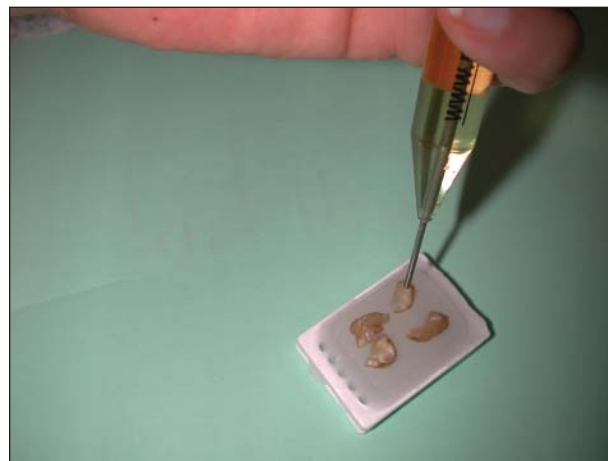
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There are several groups of biomarkers that may lend themselves to application in diagnostic and prognostic studies. Among the proliferation markers, Ki-67 is expressed in G1, S, G2 and M phase of the cell cycle but not in G0 phase.<sup>5,6</sup> Thus, it indicates cell replication but not necessarily malignant transformation and is therefore of value for cervical preneoplasia only in combination with other markers.<sup>5-7</sup> Minichromosome maintenance proteins play a role in early DNA replication.<sup>8</sup> They have been shown to be overexpressed in aberrant S-phase induction and may be a marker for high grade dysplasia.<sup>9,10</sup> The apoptosis-related marker survivin<sup>11</sup> was recently tested for cervical preneoplasia and found useful.<sup>12</sup> Most promising and numerous are cell cycle-related markers,<sup>2,5,13</sup> such as cyclin D, E and B and p16.<sup>4,13-15</sup> Finally, there have been attempts to use the viral oncogenes E6 and E7 directly as indicators for malignant transformation.<sup>16-18</sup> Further evaluation of these and other newly emerging markers in meaningful clinical settings will be necessary to clarify the potential role of these new molecular tools.

Tissue microarrays provide a means for rapid, large-scale molecular analysis of tissue specimens, providing material for various DNA, RNA and protein targets. Although the potential of combining multiple tissue specimens into a single block was recognized by Battifora<sup>19</sup> in 1986, only the refinements of the technique by Kononen et al,<sup>20-22</sup> in 1998 and afterwards led to its rapid introduction into clinical practice. In short, morphologically representative regions of archived formalin-fixed and paraffin-embedded tissue blocks are sampled with a core needle with a diameter of 0.6–2.0 mm.<sup>23,24</sup> These tissue cores from the “donor” block are then arrayed in a new “recipient” paraffin block (45 × 20 mm) using a specific



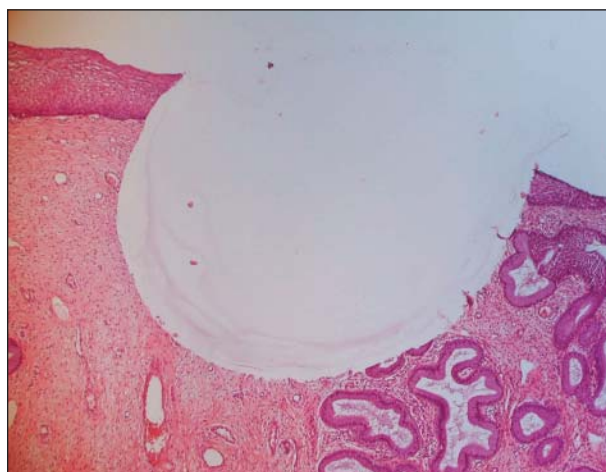
**Figure 1** Coring a conization specimen with the 0.6-mm coring needle.

instrument. As a result, up to 200 core biopsies and more from various tumors can be sampled and arrayed in a single tissue block, thus allowing rapid and efficient evaluation of new markers.<sup>25</sup> Tissue microarrays of invasive lesions have become a widespread tool in histopathology<sup>26,27</sup> not only for characterization of lesions but also for quality control.<sup>28,29</sup> There have been only a few reports on tissue microarrays of in situ lesions.<sup>9,30</sup>

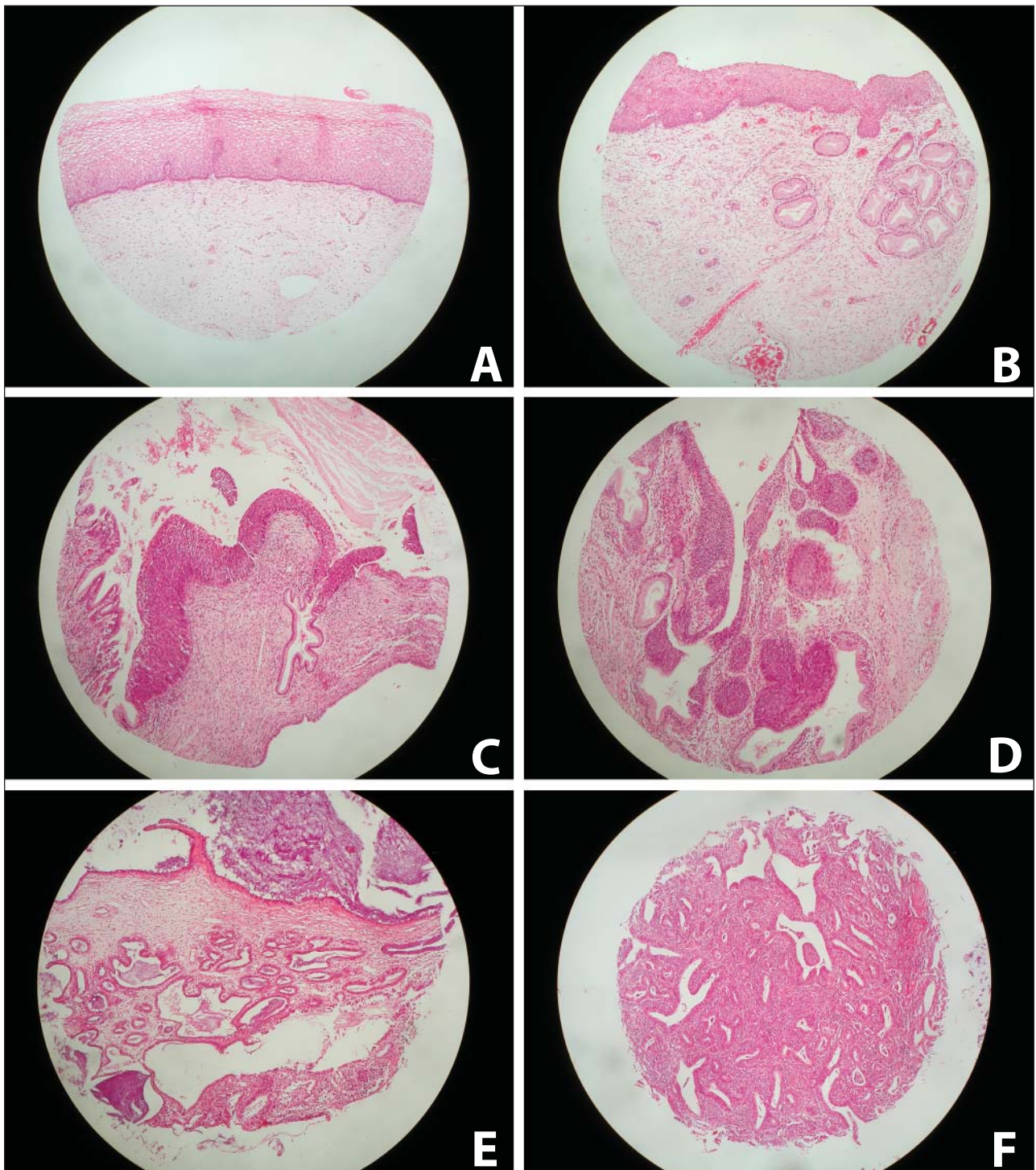
The purpose of this study was to examine the technical feasibility of constructing microarrays of precursor lesions of cervical carcinoma because the testing of markers in cervical carcinogenesis is applied mainly in the area of preinvasive lesions.

**Table 1** Original Diagnosis and Incorrect and Inadequate Cores of 240 Cervical Lesions Sampled for Tissue Microarrays

Diagnosis	n	Incorrect diagnosis	Inadequate material
Normal squamous epithelium	51	11	4
Normal glandular epithelium	44	5	4
Metaplasia	29	1	
CIN 1	13	2	1
CIN 2	21	4	1
CIN 3	55	5	6
Adenocarcinoma in situ	5	—	—
Invasive squamous carcinoma	14	—	—
Invasive adenocarcinoma	8	—	—
Total	240	28	16



**Figure 2** Section of the squamocolumnar junction after removal of a tissue core. Note the remaining carcinoma in situ at the right margin (H-E, ×000).

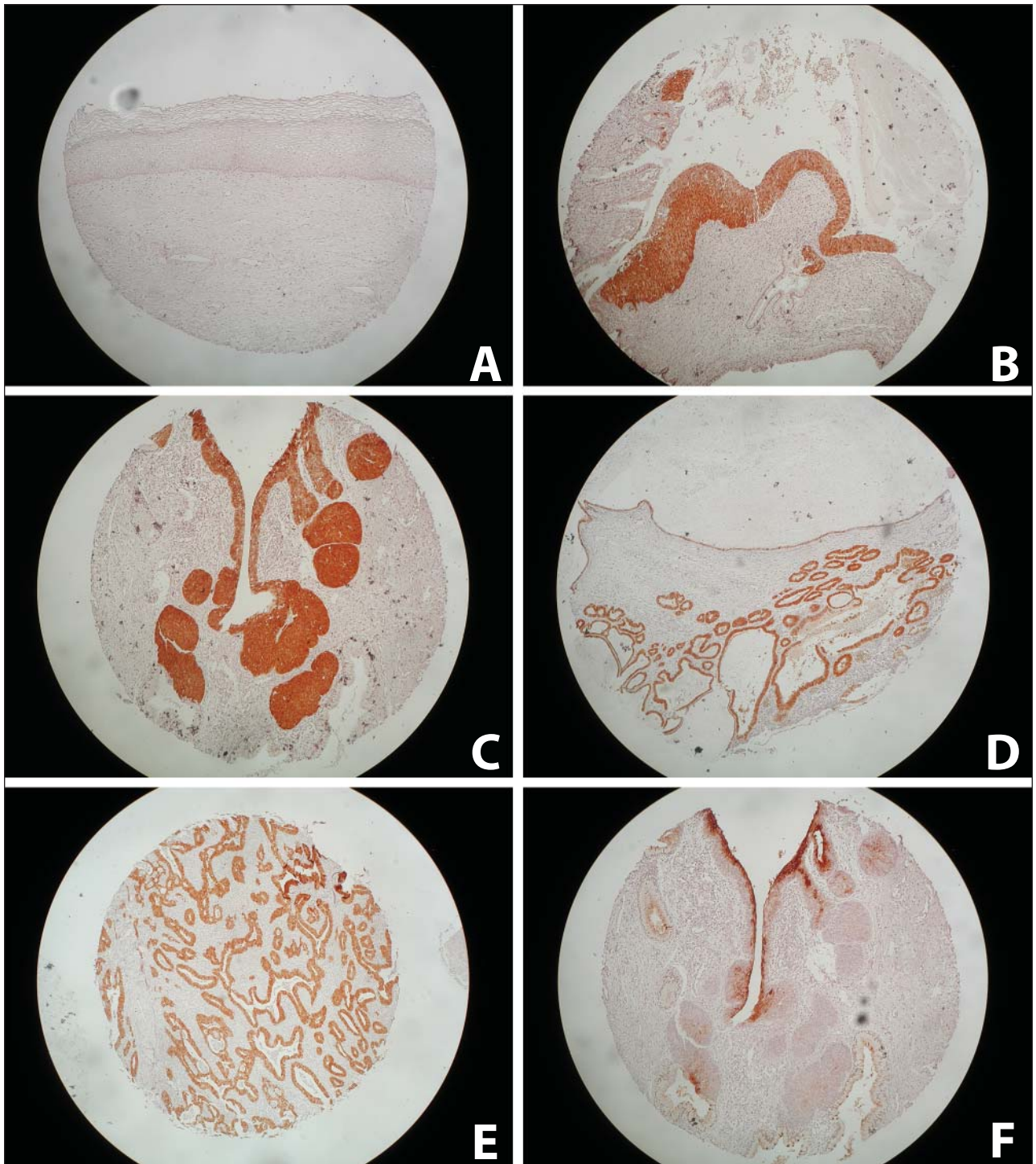


**Figure 3** (A) Tissue core of normal squamous epithelium (H-E,  $\times 000$ ). (B) Tissue core of squamous metaplasia (H-E,  $\times 000$ ). (C) Tissue core of severe dysplasia (CIN 3) (H-E,  $\times 000$ ). (D) Tissue core of severe dysplasia (CIN 3) with extensive endocervical gland involvement (H-E,  $\times 000$ ). (E) Tissue core of adenocarcinoma in situ (H-E,  $\times 000$ ). (F) Tissue core of invasive adenocarcinoma of the cervix (H-E,  $\times 000$ ).

### **Materials and Methods**

Archival tissue blocks of 240 cervical lesions sampled by biopsy, conization or hysterectomy obtained be-

tween 2003 and 2005 were selected and retrieved together with the corresponding tissue sections stained with hematoxylin-eosin (H-E). A variety of lesions,



**Figure 4** (A) Tissue core of normal squamous epithelium, negative for p16. Compare with Figure 3A (—, ×000). (B) Tissue core of CIN 3, positive for p16. Compare with Figure 3C (—, ×000). (C) Tissue core of CIN 3 with endocervical gland involvement, positive for p16. Compare with Figure 3D (—, ×000). (D) Tissue core of adenocarcinoma in situ, positive for p16. Compare with Figure 3E (—, ×000). (E) Tissue core of invasive adenocarcinoma of the cervix, positive for p16. Compare with Figure 3F (—, ×000). (F) Tissue core of CIN 3 with endocervical gland involvement, weakly positive for the novel marker C4.8(4/2/#1). Compare with Figures 3D and 4C (—, ×000). Courtesy of Matthias Duerst, Ph.D.

ranging from completely normal cervical tissue to invasive carcinomas, were selected, with the emphasis

on intraepithelial lesions (Table I). Areas free of artefacts, such as necrosis and cauterization, were selected

and marked on the H-E section. Orientation and correlation of the marked area with the corresponding paraffin block was crucial, and the use of a magnifying glass was helpful. A coring needle with an inner diameter of 0.6 mm was used (Figure 1). The cores were placed in labelled microtubes and sent to a processing company (Zytomed, Berlin, Germany), where the tissue arrays were created using a modified Beecher Instrument. The cores were arrayed in 10 rows of 6 samples each; as orientation marker a core of mouse muscle tissue was used.

The immunohistochemical p16 stain was performed according to the protocol by DakoCytomation (Glostrup, Denmark). The 5 µm paraffin sections were deparaffinized and rehydrated in graded alcohols. The sections were heated in a microwave oven at 90°C for 5 minutes for epitope retrieval in Tris/EDTA buffer, pH 9, followed by blocking non-specific binding sites with peroxidase blocking reagent. As primary antibody the mouse antihuman p16<sup>INK4a</sup> protein, clone E6H4 (DakoCytomation), was used; the incubation period was 30 minutes at room temperature. Visualization was achieved by using substrate chromogen solution (diaminobenzidine). Finally, the sections were stained with a hematoxylin counterstain for 2 minutes. Negative and positive controls were used.

## Results

Table I and Figures 1–4 summarize and illustrate the results. In 16 (6%) of 240 cases the material was insufficient. In 5 cases no tissue was present at all; in the remaining cases the tissue cores were so small that they lacked epithelial portions and were therefore not usable. In 28 cases (12%) the diagnosis of the core tissue in the microarray did not correspond to the original diagnosis of the donor block. In most cases there were discrepancies in the grade of cervical intraepithelial neoplasia (CIN), or supposedly normal squamous or glandular epithelium contained various grades of CIN. This is not surprising because transitions between these lesions are fluid and not always clearly delineated. Obviously, the 22 cases of invasive carcinoma could all be sampled correctly because these lesions are macroscopically well defined and recognizable.

The technique and successful coring of the transformation zone is illustrated in Figures 1 and 2. The various lesions and their p16 staining pattern are illustrated in Figures 3 and 4.

## Discussion

The development of biomarkers for human tumors is currently at the stage of very rapid development and has the potential to open completely new venues for diagnostic and therapeutic applications. The need for

reference standards, international cooperation, tumor repositories and coordination of academia, industry and regulatory agencies is recognized.<sup>31</sup> Cervical carcinogenesis has served as a model for many other organ sites; in particular, the stepwise transformation of normal cells into cancer cells has been studied extensively in cervical carcinoma. Similarly, the recognition of a viral infection as an initiating event has created unique opportunities to develop biomarkers as new tools for early cancer detection. Ultimately, the recognition and application of markers of progression may allow more accurate prediction of the behavior of preinvasive lesions of the cervix.<sup>32</sup> Other techniques, such as clonality analysis, may assist in these attempts.<sup>33</sup>

The current study confirmed that the creation of tissue microarrays of cervical intraepithelial lesions is feasible. Tissue microarrays have the potential to vastly facilitate the testing of emerging biomarkers of cervical carcinogenesis because numerous samples in a single paraffin block will produce large data in a standardized fashion. Careful attention to and comparison of the section and tissue block allow successful coring of the donor blocks. A certain rate of insufficient cores will probably be unavoidable; similarly, there will be a certain number of discrepant diagnoses. A second reading of the microarray is therefore mandatory to establish the definitive diagnosis of the various cores.

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