LUÍS OTÁVIO ZANATTA SARIAN

Expressão da Ciclo-Oxigenase-2 e do Ki67 em Lesões Precursoras do Câncer do Colo Uterino

Tese de Doutorado

ORIENTADORA: Profª. Drª. SOPHIE FRANÇOISE MAURICETTE DERCHAIN

UNICAMP
2005

LUÍS OTÁVIO ZANATTA SARIAN

Expressão da Ciclo-Oxigenase-2 e do Ki67 em Lesões Precursoras do Câncer do Colo Uterino

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do Título de Doutor em Tocoginecologia, área de Tocoginecologia

ORIENTADORA: Profª. Drª. SOPHIE FRANÇOISE MAURICETTE DERCHAIN

UNICAMP
2005

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS UNICAMP

Bibliotecário: Sandra Lúcia Pereira – CRB-8ª / 6044

Sa73e

Sarian, Luís Otávio Zanatta
Expressão da ciclo-oxigenase-2 e do KI67 em lesões precursoras do câncer do colo uterino. / Luís Otávio Zanatta Sarian. Campinas, SP: [s.n.], 2005.

Orientador: Sophie Françoise Mauricette Derchain
Tese (Doutorado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Imunohistoquímica. 2. Colo uterino. 3. Neoplasias. 4. Neoplasia intra-epitelial cervical. I. Derchain, Sophie Françoise Mauricette. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III Título.

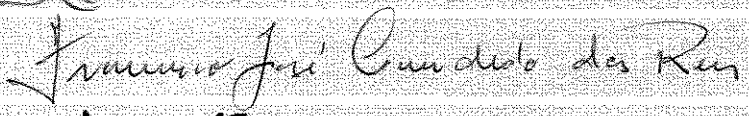
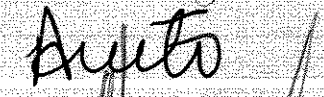
(Slp/fcm)

BANCA EXAMINADORA DA TESE DE DOUTORADO

Aluno: LUÍS OTÁVIO ZANATTA SARIAN

Orientadora: Profª. Drª. SOPHIE FRANÇOISE MAURICETTE DERCHAIN

Membros:

1. 
2. 
3. 
4. 
5. 

Curso de Pós-Graduação em Tocoginecologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas

Data: 04/11/2005

Dedico este trabalho...

ao meu pai Libarit (in memorian),
minha mãe Mariza
e ao meu irmão Paulo Henrique.

Agradecimentos

À Profa. Dra. Sophie Françoise Mauricette Derchain, pela orientação exemplar.

À Profa. Dra. Liliana Andrade, pelo apoio didático e científico durante a avaliação das lâminas de imunohistoquímica.

À Dra. Adriana Yoshida Olímpio, pela presteza e solicitude durante a pós-graduação e os trabalhos em laboratório.

Ao Prof. Dr. José Vassallo e ao Dr. Francisco Pignataro pela revisão dos artigos.

Ao Prof. Dr. Luiz Carlos Zeferino, pelo apoio institucional do Departamento de Tocoginecologia.

Ao Prof. Dr. Gustavo Antonio de Souza, pelo suporte da área de Oncologia Ginecológica do CAISM.

À Dra. Júlia Kawamura Tambascia pela leitura das lâminas de colpocitologia.

Às biólogas Denise Pita Lima, Elizabete Campos e Lúcia Carvalho, pelo processamento dos exames de Captura de Híbridos II.

Aos Professores Aarão Mendes Pinto Neto e José Antonio Simões, pelas avaliações no processo de qualificação.

À Sra. Sueli Chaves e equipe, pela revisão dos aspectos formais da tese.

À Margarete Souza Donadon, secretária da pós-graduação, pelo apoio durante os trâmites burocráticos.

À Neuza Balducci, Leandro Damião, Sônia Pianca e Pryscilla Rosa, da secretaria da área de oncologia, pelo auxílio logístico.

Às amigas Joana Bragança, Sílvia Rabello, Cláudia Inhaia, Samara Messias, Renata Gontijo, e Eliane Zambelli, pela ajuda e coleguismo durante os anos de pós-graduação.

À Dra. Adriana Salzani, pelo discernimento e companheirismo.

Este estudo foi parcialmente financiado pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processos números 00/06394-0 e 04/09309-5 e Conselho de Desenvolvimento Científico e Tecnológico (CNPq), processo número n° 307252/2004-3

A coleta de dados foi realizada no Ambulatório de Patologia Cervical do Centro de Atenção Integral à Saúde da Mulher (CAISM) da Unicamp.

O estudo teve a participação dos Departamentos de Tocoginecologia e Anatomia Patológica da Faculdade de Ciências Médicas da Unicamp; dos Laboratórios de Citopatologia, Clínico Especializado e de Patologia Experimental do CAISM.

Estrutura da Tese

Esta tese está sendo apresentada em formato alternativo, de acordo com o disposto em "*Normas, procedimentos e orientações para publicações de dissertações e teses da Faculdade de Ciências Médicas da Universidade Estadual de Campinas (2005)*". Inclui resumo e *summary*, seguidos por introdução ao tema, objetivos do projeto de pesquisa e dois artigos originais, escritos em língua inglesa - um deles submetido e outro a ser enviado para publicação. Métodos, resultados e discussão são apresentados em cada artigo. A tese é finalizada com breve discussão geral, as conclusões e as referências bibliográficas. Nos anexos foram incluídos o termo de consentimento livre e esclarecido e um painel imuno-histoquímico, ilustrando duas reações para COX-2 e duas para Ki67.

Sumário

Símbolos, Siglas e Abreviaturas

ALTS	*ASCUS-LSIL Triage Study*
ASC	Atipia de células escamosas; *Atypical squamous cells*
°C	Graus Celsius; *Celsius degrees*
CAISM	Centro de Atenção Integral à Saúde da Mulher
CHII	Captura de Híbridos II
CI	Intervalo de confiança; *Confidence interval*
CIN	*Cervical intraepithelial neoplasia*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO	Colpocitologia oncológica; *colpocytology*
COX-2	Ciclo-oxigenase-2; *Cyclooxygenase-2*
DAB	Tetra-hidrocloreto de diaminobenzidina; *Diaminobenzidine tetrahydrochloride*
e.g.	Por exemplo; *for exemple*
DNA	Ácido desoxirribonucléico; *desoxirribonucleic acid*
et al.	E outro(s), e outra(s)
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FU	Seguimento clínico; *follow-up*
G	Intervalo; *Gap*
HCII	Captura de Híbridos II; *Hybrid Capture II*

H&E	Hematoxilina e Eosina; *Hematoxylin and eosin*
HPV	Papilomavírus humano; *Human papillomavirus*
H_2O_2	Água oxigenada; *Hydrogen peroxide*
HSIL	Lesão escamosa intra-epitelial de alto grau; *High-grade squamous intraepithelial lesion*
i.e.	Ou seja; *that is*
LEEP	Excisão da zona de transformação por eletrocirurgia de alça; *Loop electrosurgical excision procedure*
LSIL	Lesão escamosa intra-epitelial de baixo grau; *Low-grade squamous intraepithelial lesion*
NIC	Neoplasia intra-epitelial cervical
OR	Razão de chances; *Odds ratio*
PBS	Solução salina tamponada com fosfato; *Phosphate buffered saline*
PC	Controle positivo; *Positive control*
PCR	Reação em cadeia da polimerase; *Polymerase chain reaction*
Pg/mcl	Picograma por microlitro; *Picogram per mililiter*
RLU	Unidades relativas de luz; *Relative Light Unit*
RNA	Ácido ribonucléico; *ribonucleic acid*
RR	Razão de risco; *Risk ratio*
SCC	Carcinoma de células escamosas; *Squamous cells carcinoma*
SIL	Lesão escamosa intra-epitelial; *Squamous intraepithelial lesion*
TBS	Terminologia do Sistema Bethesda; *Terminology of the Bethesda System*
Unicamp	Universidade Estadual de Campinas
USA	Estados Unidos da América; *United States of America*

Resumo

Objetivos: Avaliar a expressão da ciclo-oxigenase-2 (COX-2) em lesões escamosas da cérvice uterina e suas relações com a expressão nuclear do Ki67, a detecção do papilomavírus humano (HPV) e com a persistência/recorrência de neoplasia intra-epitelial cervical (NIC) após conização diatérmica. **Sujeitos e métodos:** Este foi um estudo de coorte, com análise intermediária em corte transversal, para o qual foram selecionadas mulheres submetidas à conização diatérmica para o tratamento de anormalidades cervicais, entre fevereiro de 2001 e abril 2004. O estudo é apresentado em dois artigos: o primeiro consiste de análise transversal, incluindo os espécimes cirúrgicos de 164 mulheres submetidas à conização. Foram avaliadas a expressão citoplasmática da COX-2 e a expressão nuclear do Ki67, determinadas por imuno-histoquímica, segundo a gravidade da lesão cervical e a detecção do HPV de alto risco oncogênico, realizada através de Captura de Híbridos II. No segundo artigo, com análise longitudinal, foram incluídas 104 mulheres com NIC, seguidas por até 36 meses após conização diatérmica, para avaliar possíveis relações entre a expressão da COX-2 e Ki67, comprometimento das margens do cone e detecção do HPV (durante o seguimento), com a persistência/recorrência

da NIC. **Resultados:** No primeiro artigo pode-se observar que não houve diferença na expressão citoplasmática da COX-2 relacionada à gravidade da lesão cervical, à expressão do Ki67 e à detecção do HPV de alto risco. Houve maior expressão de Ki67 nos espécimes com NIC 3. No segundo artigo, 14 casos de recorrência/persistência da NIC foram detectados durante o seguimento. A expressão citoplasmática da COX-2 e a expressão nuclear do Ki67 foram semelhantes nos casos com ou sem recorrência/persistência, e apenas a detecção do HPV de alto risco oncogênico durante o seguimento esteve associada ao maior risco de persistência/recorrência da NIC (Razão de risco 7,6; Intervalo de confiança 95% 2,1-28,6). **Conclusões:** A expressão citoplasmática da COX-2 não esteve relacionada à gravidade da lesão cervical, à expressão de Ki67 ou à infecção pelo HPV. As expressões da COX-2 e do Ki-67 não se relacionaram com a persistência/recorrência da NIC em até 36 meses de seguimento.

Summary

Objectives: To assess the expression of cyclooxygenase-2 (COX-2) in squamous lesions of the uterine cervix and its possible relations to nuclear expression of Ki67, Human papillomavirus (HPV) detection and to the persistence/recurrence of cervical intraepithelial neoplasia (CIN) after diathermic conization. **Subjects and methods:** This was a prospective study with intermediate cross-sectional analysis, in which women subjected to diathermic conization due to cervical abnormalities were selected between February 2001 and April 2004. The first article is a cross sectional study, including surgical specimens of 164 women subjected to conization. Cytoplasmic expression of COX-2 and nuclear expression of Ki67, ascertained through immunohistochemistry, was evaluated according to cervical lesion grade and high-risk HPV detection through Hybrid Capture II. In the second article, 104 women with CIN were included, followed-up for 36 months after diathermic conization, in order to assess possible relation between COX-2 and Ki67 expression, adjusted by the cone margin status and high-risk HPV detection during follow-up with persistent/recurrent CIN. **Results:** Cross-sectional analysis of baseline data disclosed that COX-2 protein expression did not differ in relation to grade of cervical lesion, Ki67 expression and high-risk HPV detection. A higher

expression of Ki67 was associated exclusively with CIN 3. In the follow-up series, fourteen cases of recurrent/persistent CIN were detected after diathermy conization. Persistence/recurrence of CIN were not related to cytoplasmic COX-2 and nuclear Ki67 expression, but had a positive association with high-risk HPV detection during follow-up (Risk Ratio 7.6; 95% confidence interval 2.1-28.6). **Conclusion:** The cytoplasmic expression of COX 2 did not correlate with cervical lesion grade, nuclear Ki67 expression and high-risk HPV detection. COX-2 and Ki67 expression was not related to persistence/recurrence of CIN.

1. Introdução

As neoplasias intra-epiteliais cervicais (NIC) grau 2 ou 3 são as lesões precursoras do câncer do colo do útero. Vinte e dois por cento das mulheres com NIC 2 podem evoluir para NIC 3, e 5% daquelas com NIC 3 podem evoluir para câncer (Östor, 1993). Essa evolução é lenta, permitindo o diagnóstico da doença em fases pré-invasoras e seu tratamento. A técnica mais empregada para retirada das NIC 2 e NIC 3 é a conização com bisturi ou com alça diatérmica. Embora a conização com bisturi tenha sido largamente empregada até o final da década de 1980, rapidamente a técnica com alça diatérmica ganhou espaço e confiabilidade, tornando-se a preferida em muitos serviços. Como principal vantagem, a conização diatérmica pode ser realizada em ambulatório e com anestesia local, enquanto a conização com bisturi só pode ser realizada em centro cirúrgico e com bloqueio espinhal ou anestesia geral. Realizadas com técnica apropriada e com pessoal bem treinado, as taxas de cura são semelhantes para ambas as técnicas (Duggan et al., 1999).

Existe risco de recidiva ou doença residual e o seguimento das mulheres tratadas com conização diatérmica deve ser rigoroso. Alguns autores referem doença residual em até 30% dos espécimes de histerectomia após conização (Lin et al., 2001). Outros, após tratamento conservador, relatam recidivas em 5% a 10% dos casos em até 36 meses de seguimento (Flannely et al., 1997). Se, por um lado, a doença pode estar curada mesmo nos casos em que as margens do espécime de conização estavam comprometidas, por outro, mulheres com remoção completa da NIC podem apresentar recidivas (Paraskevaidis et al., 2003). Em geral, adota-se como protocolo de seguimento pós-operatório consultas semestrais, por dois ou três anos, com exame ginecológico, colposcopia e coleta de colpocitologia oncológica (CO). Entretanto, é importante considerar que a CO pode ser normal mesmo em casos onde houve confirmação histológica de recidiva, o que faz ser relevante a pesquisa de métodos mais sensíveis para acompanhar mulheres tratadas por conização diatérmica (Flannely et al., 2001; Tangtrakul et al., 2002).

Com o advento de técnicas biomoleculares que permitiram a identificação do papilomavírus humano (HPV), vários estudos estabeleceram papel causal para este vírus na evolução da NIC (Wacholder 2003). Como conseqüência, crescente interesse vem sendo dirigido à possível contribuição que a detecção do HPV possa ter no seguimento de mulheres tratadas da NIC. De fato, a permanência ou recorrência da NIC acontece em mulheres com infecções persistentes após o tratamento (Nagai et al., 2000; Nobbenhuis et al., 2001). Três estudos recentes confirmaram que o teste para HPV pode ser importante na determinação de

mulheres com maior risco de apresentar recidivas ao longo do seguimento (Costa et al., 2003; Zielinski et al., 2003; Sarian et al., 2004).

Para que o HPV exerça seu efeito nas células da cérvice uterina, seus oncogenes devem afetar elementos do ciclo de divisão celular, ou simplesmente "ciclo celular". Este, esquematicamente, é dividido em fases distintas que culminam na mitose, fase em que ocorre a divisão do material nuclear e que conduz à bipartição da célula. A replicação do ácido desoxirribonucléico (DNA) ocorre na fase S (de Síntese), sendo o intervalo entre o final da mitose e o início da fase S chamado de fase G1 (G = *Gap*), e o intervalo entre o final da síntese de DNA e o início da mitose chamado de fase G2. Durante a fase G1, a célula prepara seu aparato biológico para viabilizar o processo de síntese de DNA e, no momento apropriado, dá início à síntese do material nuclear. Em determinadas circunstâncias, a célula em G1 pode entrar em estado de repouso, geralmente chamado de G0, fase esta em que pode permanecer por dias, semanas ou mesmo anos, antes de retornar ao processo de proliferação (Alberts et al., 1994).

Os oncogenes virais do HPV atuam em duas etapas distintas do ciclo celular: no controle da passagem da fase G1 para S e na regulação da entrada da célula em apoptose (Wentzensen et al., 2004). Alterações no controle do ciclo celular e da apoptose resultam em maior número de células em proliferação, sendo que a proporção de células neste estado é um importante marcador de gravidade das neoplasias. A quantidade de células em estado proliferativo pode ser indiretamente medida através da detecção de um antígeno nuclear expresso no final da fase G1, toda a fase S e início de G2, chamado Ki67. Estudos anteriores

associaram o crescimento da proporção de células coradas pelo Ki67 à piora da gravidade da NIC e de outras neoplasias (Keating et al., 2001; Kruse et al., 2001).

Em paralelo ao estudo de marcadores moleculares relacionados ao controle do ciclo celular e da apoptose, há aquele dedicado a outras proteínas que provavelmente exerçam influência no processo de perda da diferenciação celular, proliferação anômala e comportamento clínico das neoplasias. Recentemente, alguns estudos demonstraram a maior expressão da enzima prostaglandina-endoperoxidase sintetase 2, ciclooxigenase-2 ou, simplesmente, COX-2, em tumores de mama, cólon-reto, estômago, cabeça e pescoço, pulmão e colo do útero (Lim et al., 2000; Ryu et al., 2000; Shamma et al., 2000; Kulkarni et al., 2001). Em condições normais, a COX-2 é responsável pela transformação do ácido aracdônico em prostaglandina H_2, fase importante da resposta inflamatória. Suspeitou-se de sua participação na carcinogênese após terem sido detectadas reduções de 40% a 50% no risco para o desenvolvimento de carcinoma colorretal (DuBois et al., 1996) e, em menor intensidade, também para câncer de próstata e mama (Norrish et al., 1998), entre pessoas que usavam inibidores da COX para tratamento de condições inflamatórias crônicas.

Hoje se acredita que a COX-2 esteja associada à inibição da apoptose e ao aumento da adesão à matriz extracelular, além de facilitar a metastatização, invasividade tumoral e neo-angiogênese (Tsujii et al., 1998). Especificamente em relação ao câncer do colo do útero, a maior expressão da enzima teve associação com o prognóstico, ao estar relacionada com pior resposta à radioterapia e maior resistência à quimioterapia. Entretanto, o comportamento da expressão

da COX-2 em lesões pré-invasoras do colo do útero ainda não foi totalmente estabelecido, e os dados a esse respeito ainda são muito escassos na literatura. Farley et al. (2004), estudando 62 mulheres tratadas com conização diatérmica, encontraram que a maior expressão da COX-2 nas áreas com NIC ou nas margens de conização esteve significativamente associada com a persistência ou recidiva da doença, mas estes resultados não foram debatidos por outros autores.

A avaliação dos marcadores moleculares em lesões escamosas pré-invasoras do colo do útero trará informações relevantes para o entendimento do comportamento clínico da NIC após a conização diatérmica. Este estudo faz parte de uma linha de pesquisa, financiada pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e pelo Conselho Nacional de Pesquisa (CNPq), que pretende avaliar as "Recidivas da NIC após conização diatérmica em mulheres com infecção pelo HPV, em função da expressão de proteínas marcadoras da regulação do ciclo celular, da apoptose e com participação na invasividade tumoral e neo-angiogênese". Os conhecimentos atuais a respeito dos fatores que levam à recidiva da NIC são insuficientes, o que torna necessária a aquisição de informações para melhor determinar o risco de recidiva entre mulheres com infecção persistente pelo HPV. Nesta tese serão abordadas a expressão citoplasmática da COX-2 e a expressão nuclear do Ki67.

2. Objetivos

2.1. Objetivo geral

Avaliar a expressão da COX-2 em lesões escamosas da cérvice uterina e suas possíveis relações com a expressão nuclear do Ki67, a detecção do HPV de alto risco oncogênico e a persistência/recorrência de NIC após conização diatérmica.

2.2. Objetivos específicos

- **Artigo 1:** Avaliar a expressão da COX-2 em relação à gravidade da lesão cervical, expressão nuclear do Ki67 e à detecção do HPV de alto risco oncogênico.

- **Artigo 2:** Avaliar a freqüência de recidivas da NIC após conização com alça diatérmica em função da expressão citoplasmática da COX-2, da expressão nuclear do Ki67, das margens do cone e da infecção pelo HPV de alto risco oncogênico durante o seguimento.

3. Publicações

Artigo 1 – Expression of Cyclooxygenase-2 (COX-2) and Ki67 as related to disease severity and HPV detection in squamous lesions of the cervix.

Artigo 2 – HPV persistence, expression of cyclooxygenase 2 (COX-2) and Ki67 as related to the risk of cervical intraepithelial neoplasia persistence/recurrence after diathermic conization.

3.1. Artigo 1

Expression of Cyclooxygenase-2 (COX-2) and Ki67 as related to disease severity and HPV detection in squamous lesions of the cervix

Luis Otavio Sarian[1]
Sophie Françoise Derchain[1]
Adriana Yoshida[1]
José Vassalo[2]
Francisco Pignataro[2]
Liliana Aparecida Lucci De Angelo Andrade[2]

1. Department of Obstetrics and Gynecology; 2. Department of Pathology, Universidade Estadual de Campinas (UNICAMP)

Corresponding author.
Sophie F. M. Derchain, MD, PhD
Rua Antônio Hossri, 629 – Cidade Universitária
13083-370 – Campinas – SP.
Fone: +55-19-37889305 / Fax: + 55-19-3289 5935
E-mail: derchain@supernet.com.br

Running headline: COX-2 and Ki67 in cervical lesions.
Key words: MIB-1; cervical intraepithelial neoplasia, cancer, cervix, immunohistochemistry

Artigo submetido para publicação no Gynecologic Oncology

Abstract

Objectives: To assess the expression of COX-2 and Ki67 in cervical squamous lesions in relation to disease severity and Human papillomavirus (HPV) detection. **Subjects and methods:** For this cross-sectional study, 165 women subjected to Loop Electrosurgical Excision Procedure (LEEP) of the cervix have been enrolled, between February 2001 and April 2004. All patients undertook pelvic examination, including colposcopy and collection of samples for Hybrid Capture II. Pathological assessment disclosed: 9 cases of normal epithelium/cervicitis, 33 cervical intraepithelial neoplasia (CIN) 1, 21 CIN 2, 95 CIN 3 and 7 invasive squamous cell carcinoma. COX-2 and Ki67 protein expression was determined with immunohistochemistry. COX-2 immunoreactivity grading was based on the German ImmunoReactive score. The continuum percentage of positive cells was used for the assessment of nuclear Ki67 expression. **Results:** Expression of COX-2 did not correlate with disease severity and with Ki67 expression. The HPV detection rates did not differ significantly across COX-2 protein expression strata, ranging from negative to strong expression. Ki67 expression, however, was higher in the CIN 3 group (p=0.001) as compared to the specimens rendered as normal/cervicitis. **Conclusions:** COX-2 protein expression did not correlate with disease severity or Ki67 expression.

Introduction

Cancer of the uterine cervix is still one of the prime concerns in public health, mainly in the developing world. The disease is preventable and has its etiology firmly correlated with Human papillomavirus (HPV) infection [1]. The treatment of its pre-invasive form, the cervical intraepithelial neoplasia (CIN), can be easily accomplished. In recent years, several biologic markers or indexes have been studied as potential tools to determine the prognosis and biological behavior of various types of neoplasia, and immunohistochemistry (IHC) is probably the most affordable and simple technology to detect many such biomarkers. This fact prompted investigators to develop and test antibodies in a widespread range of neoplasic lesions, and gynecological pathology has not escaped this tendency [2,3].

Oncogenes and cell-cycle regulators suggested to play a role in genesis of cervical cancer include c-erb-2/neu, p27, p53 and p16INK4a and Ki67 [4]. In very simple terms, the expression of these markers is affected by the disrupting effects of HPV E6 and E7 oncogenes on the cell-cycle control mechanisms, leading to uncontrollable cell proliferation. Ultimately, cell multiplication can be assessed by detecting the antigen Ki67, expressed in the nuclei of proliferating cells. The rate-limiting enzyme involved in the conversion of arachidonic acid to prostanglandins in inflammatory processes, Cyclooxygenase-2 (COX-2), has also been shown to correlate with carcinogenesis in humans [5]. Firstly studied in esophageal squamous tumors, COX-2 has also been demonstrated to be overexpressed in breast, stomach, colon and cervical neoplasms as well. It is suggested that COX-2, besides its participation in inflammatory responses,

might induce tumor proliferation and spread by enhancing mitogenesis, reducing cellular adhesion and immune surveillance [6]. In cervical cancer, comparative studies found that COX-2 might be prognostic, relating to a diminished response to radiotherapy, in terms of survival, and to an augmented resistance to chemotherapy [7, 8]. However, the behavior of COX-2 in pre-invasive cervical lesions is still under investigation, and its expression has been suggested to increase in parallel to the grade of CIN, and possibly to have a potential in help predicting CIN recurrence after conservative treatments (e.g. diathermic or cold-knife conization) [9]. Nevertheless, the number of studies available in literature remains limited and discrepant results have been reported.

In order to shed further light on the understanding of COX-2 in the pre-invasive forms of squamous cervical neoplasia, we decided to conduct this study, aimed at assessing the expression of COX-2 in different grades of squamous lesions of the uterine cervix and its possible relations to nuclear expression of Ki67 and human papillomavirus (HPV) detection.

Materials and methods

Selection of the women and examination routine

For this cross-sectional study, 165 non-consecutive women subjected to loop electrosurgical excision procedure (LEEP) of the cervix were enrolled. Women had been referred to the colposcopy clinics of Universidade Estadual de Campinas, Brazil, due to abnormal findings in their cervical cytology smears (Pap tests). All patients were subjected to an interview, addressing clinical and

socio-demographic concerns, and shortly afterwards to a thorough pelvic examination, including colposcopy and collection of samples for Hybrid Capture II (HCII). The decision to perform diathermic conization as the primary treatment was made on the basis of the cytology result coupled with the clinical/colposcopic aspect of the cervix. Enrollment began in February 2001 and ended April 2004. The study has been approved by the local Ethics Committee, and all patients signed the Informed Consent Form.

HPV detection

The specimens for HCII were tested with probe B (high-risk HPVs: types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) [10] and the tests were classified at the relative light unit/positive control (RLU/PC) ratio (RLU of specimen/mean RLU of two positive controls) of 1pg/ml or greater. These RLU/PC ratios provided an estimate of the amount of HPV-DNA in the specimens, i.e. the viral load in the sample. The storage of the specimens and all reagents as well as conduction of the tests took place at the Campinas State University Medical School Hospital Laboratory, following the manufacturer's instructions (*Digene Diagnostics Inc., USA*).

Histology

Histological samples consisted of 165 diathermic conization specimens, which were fixed in 10% phosphate buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Samples for histology were analyzed according to the World Health Organization criteria's [11] and classified as

normal/cervicitis (9 cases), CIN1 (33 cases), CIN2 (21 cases), CIN3 (95 cases) and invasive squamous cell carcinoma (7 cases).

Immunohistochemistry (IHC)

IHC reactions were performed on slides obtained from the tissue blocks that harbored the most significant lesion, as detected with H&E assessment. Five micrometer sections were obtained from the tissue blocks and transferred to silanized glass slides and dried overnight at 37° C. The sections were deparaffinized using a sequence of xylene, followed by washes in graded ethanol and phosphate buffered saline (PBS; pH7.4). Pretreatment of the slides consisted of microwaving for 15 minutes in 10mmol/L sodium citrate (pH 7.0). After washing the slides with PBS, endogenous peroxidase was blocked by 3% hydrogen peroxide (H_2O_2), by three sequential incubation baths, 3 min each. Samples were then incubated for 30 minutes in a humid chamber at 37°C with the antibodies: anti-COX-2 (1:50 dilution) or Ki67 (1:100 dilution). Afterwards, slides were incubated overnight at 4°C. Slides were then incubated with biotinylated linked secondary antibody (DAKO A/S, Copenhagen, Denmark) for 30 minutes and with a solution of avidin biotinylated peroxidase. For slides development a diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate was used, for 6 minutes at 37°C. The slides were washed with water and counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted with resin Entellan®. Slides with colitis known to be positive for the

COX-2 were used as positive controls. The corresponding sections without the primary antibody were employed as negative controls.

Assessment of IHC slides

Reading of all slides was performed by one investigator, who was blinded to the H&E diagnosis rendered by a pathologist and to the HPV status of the patients. Digitalized photographs of high power fields (400x) were taken from hot-spots of reaction with a Nikon COOLPIX Camera 995, and then images were ported to a Personal-Computer based software for histological analyses (Imagelab 2000).

COX-2 protein expression

COX-2 immunoreactivity grading was based on the German ImmunoReactive score [12]. Firstly, staining intensity in the cytoplasm was rated on a scale from 0 to 3, with 0 being no staining at all, 1 weak staining, 2 moderate and 3 strong staining. Then, positive and negative cells were counted, with no less than 500 cells as the minimum acceptable count number. The percentage of positive cells was then scored as: no staining as 0; 1-10% as 1; 11 to 50% as 2; 51 to 80% as 3 and 81% to 100% as 4. The final score was calculated by multiplying the score obtained with the staining intensity by that derived from the percentage of positive cells, achieving theoretical results ranging 0 to 12. A final score of 0 was regarded as negative, 1-4 as weak, 5-8 as moderate and 9 to 12 was considered as strong immunoreactivity. COX-2 protein expression was negative in 14 (8.5%) histological specimens, whereas

53 (32.1%) lesions presented with weak expression, 44 (26.6%) with moderate and 54 (32.7%) with strong expression of the antigen. For statistical purposes, two groups were formed: negative and weak immunoreactivity (67 women); moderate and strong immunoreactivity (98 women).

Ki67 nuclear expression

A continuum percentage of cells expressing Ki67 in their nuclei was used for the assessment of cell proliferation rate. A minimum count of 500 nuclei was mandatory. Nuclear staining of basal cells was considered as the internal positive control of reaction. Specimens of lung adenocarcinoma, known to be strongly positive for Ki67, were used as external reaction positive controls.

Statistical analysis

A logistic regression model, adjusting for the pathological categories, has been applied to evaluate a possible relation between HPV detection and COX-2 expression. Odds ratios (OR) were calculated by exponentiation of the correlation coefficients. A generalized linear model, also adjusted for the pathological assessment, has been used to determine possible correlations between Ki67 expression – reflecting the cell proliferation rate - and COX-2 protein expression. Another logistic regression model has been fit - adjusted for the HPV status of the women - to investigate a possible relation between histological grade of the disease and COX-2 protein expression. The evaluation of Ki67 expression as related to the pathological categories has been carried out through a generalized linear model, with adjustment for the HPV detection. Data

were recorded in an OpenOffice® spreadsheet and statistical treatment has been performed with the R *Environment* [13] statistical software package, set to 95% confidence intervals (95%CI).

Results

Mean women's age was 36.5 years (90% central range 23.5-53.8 years; data not shown). Ninety-eight women (59.4%) had lesions that expressed COX-2 'significantly', i.e with moderate to strong expression of the protein. Negative/weak expression of COX-2 was present in 6 out of 9 (66%) women with normal histology/cervicitis, 20 of 33 (60%) with CIN 1, 7 of 21 (33%) of those with CIN 2, 62 of 95 (65%) in CIN 3 and 3 of 7 in cancer specimens. Expression of COX-2 was not significantly different across histological strata. Nuclear Ki67 expression increased in parallel to severity of the disease, being significantly higher in CIN3 (p=0.001; Table 1).

One hundred thirty-eight women (83.6%) were HPV positive at enrollment. HPV detection ranged from 55.6% in women with normal/cervicitis final pathological diagnosis to 100.0% among those with cancer. Women with CIN 2 presented with 81.0% prevalence of HPV infection, as detected by HCII, and women diagnosed with CIN 3 had an overall HPV prevalence of roughly 97% (data not shown).

The HPV detection rates did not differ significantly across COX-2 expression strata (OR=1.5; 95%CI 0.6 to 4.2). Ki67 immunoreactivity ranged from mean expression of 37.6% among cases negative/weak for COX-2, to 39.0% in those with moderate/strong expression of COX-2. However, there were

no statistical differences in cell proliferation rates as related to COX-2 expression (p=0.87) (Table 2).

Discussion

Expression of COX-2 and its possible relation to prognosis has been the prime focus of several studies reporting on invasive cervical lesions [7, 8, 14-16]. Data and analysis on pre-invasive lesions, however, remains scant in the recent literature [9, 17]. In the present series, expression of COX-2 has been studied in a relatively large sample of 165 cervical conization specimens, yielding additional evidence that can possibly feed the debate on this relatively new biological marker. Importantly, the present results were obtained with an affordable and widely mastered technology, allowing the discussion on the eventual application of COX-2 testing in the routine clinical practice.

COX-2 expression did not correlate with the variables in study, namely the CIN grade, HPV status or Ki67 expression. Interesting, however, is the fact that approximately 60% of the specimens harbored a significant expression of the antigen. Using real-time polymerase chain reaction (RT-PCR), a Chinese study group [18] reported that COX-2 mRNA was not detected in 20 normal cervix tissue samples (0-0.045 pg of mRNA), whereas 17 out of 20 cases of cervicitis did express COX-2 mRNA, being that three of such cases were detected with elevated amounts of mRNA. Of note, there was no difference between COX-2 mRNA expression in specimens of inflammation compared to those harboring CIN. In that same study, 36 of 40 cancer specimens were considered as having "very high" amounts of COX-2 mRNA. The information

provided by Dai et al. may partially explain some of the present findings, because normal and inflammatory (cervicitis) were under the same label. Given that the entire set of women included in the present study were referred to diathermic conization because of an abnormal cytology, it may be sensible to state that the majority of women, even those without CIN in their conization specimens, might have an underlying inflammatory process when conization was performed. It has been shown before that HPV infection elicits inflammatory responses, and that other infectious states of the cervix might provoke abnormalities in Pap smears.

Many authors ascribe a pivotal role in oncogenesis to COX-2, by mediating angiogenesis, immune surveillance, cell adhesion and apoptosis [19,20]. The enzyme expression has been shown to correlate well with levels of the cyclin-dependent kinase inhibitor p27, with COX-2 levels increasing as neoplasia progresses, and the contrary occurring with p27 [9]. Because p27 is important in regulating the G1/S transition, its reverse association with COX-2 expression suggests that this enzyme levels are much likely to be affected by the disruptive mechanisms of the cell cycle that lead to uncontrollable proliferation in neoplasia. Ki67 nuclear staining is the most widely used technique to assess cell proliferation. Therefore, the combined use of COX-2 and Ki67 in different slides obtained from the same tissue samples allowed the assessment of a possible relation between proliferation rate and COX-2 protein expression. In the present series, however, higher proliferating rates were related only to the histological diagnosis of CIN 3 and cancer, and there was no relationship between Ki67 and COX-2 protein expression. Again, inflammatory

processes, inherently linked to the pathogenesis of CIN, may justify the relatively high percentage of positive Ki67 cells in normal/cervicitis (up to 41%) and CIN1 (up to 50%) specimens.

Indeed, the accelerated cell growth encountered in most of the present cases reflects a severely disrupted growth regulation of the squamous epithelium. It is important to consider that both Ki67 and COX-2 were evaluated in the entire epithelium, differently, for example, from the immunohistochemical assessment of p27 expression analysis carried out by Shiozawa et al [17]. In that study, the marker (p27) used to assess stability of the cell growth control system was evaluated only in proliferating cells (i.e. those expressing Ki67). In the present study, COX-2 protein expression was measured throughout the epithelial layers, regardless of the Ki67 status of the cells. With this approach, cells in different cell-cycle status were regarded as a homogenous group, which might lead to different results as compared to the findings by Shiozawa et al. For instance, it is known that cells in the upper layers of the normal epithelium are generally quiescent and do not express Ki67, whereas basal cells usually do express it, because of the characteristic proliferating function of basal layer. This heterogeneous antibody expression might occur with COX-2 as well, but the present study was not designed to detect these eventual differences in COX-2 expression on the basis of Ki67 reaction.

A major strength of this study is the fact that the entire series has been tested for HPV prior to diathermic conization. By knowing the HPV status, the authors were able to fit regression models that took into account not only the variables under scrutiny (COX-2, Ki67 and disease severity), but also the

presence of the causal agent of the disorder. However, the high prevalence of HPV across histological strata hampered the analysis on COX-2 expression and HPV positivity. This study's characteristic may be attributed to the selection criterion of only including women with abnormal Pap smears, which reduced dramatically the number of specimens with epithelium not yet disturbed by inflammatory processes.

The present study corroborates the previous finding that COX-2 expression is abundant in epithelial cervical lesions. However, COX-2 detection was not related to disease severity or to the growth rate, as ascertained by the Ki67 staining. Therefore, COX-2 applicability in clinical practice is still an investigational matter, and prospective cohort studies assessing the rate of pre-invasive disease recurrence following conservative treatments are necessary. A study in this respect is currently under way in our cervical pathology clinics.

Acknowledgements

To Denise Pitta and Lucia Fagian for technical assistance in HPV detection. Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processes number 00/06394-0 and 04/09309-5 and Conselho de Desenvolvimento Científico e Tecnológico (CNPq), process number 307252/2004-3.

References

1. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003; 348:518-527.
2. McCluggage WG. Recent advances in immunohistochemistry in gynecological pathology. Histopathology 2002; 40:309-326.
3. McCluggage WG. A critical appraisal of the value of immunohistochemistry in diagnosis of uterine neoplasms. Adv Anat Pathol 2004; 11:162-171.
4. Tringler B, Gup CJ, Singh M, et al. Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia. Human Pathol 2004; 35(6):689-96.
5. Shamma A, Yamamoto H, Doki Y, et al. Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. Clin Cancer Res 2000; 6:1229-38.
6. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999; 19:790816.
7. Gaffney DK, Holden J, Zempolich K, Murphy KJ, Dodson M. Elevated cyclooxygenase-2 expression correlates with diminished survival in carcinoma of the cervix treated with radiotherapy. Int J Radiat Oncol Biol Phys 2001; 49:1213-7.
8. Ferrandina G, Lauriola L, Distefano MG, et al. Increased cyclooxygenase-2 expression is associated with chemotherapy resistance and poor survival in cervical cancer patients. J Clin Oncol 2002; 20:973-81.

9. Farley J, Uyehara C, Hashiro G, Belmap C, Birrer M, Salminen E. Cyclooxygenase-2 expression predicts recurrence of cervical dysplasia following loop electrosurgical excision procedure. Gynecol Oncol 2004; 92: 596-602.

10. Nindl I, Lorincz A, Mielzynska I, et al. Human papillomavirus detection in cervical intraepithelial neoplasia by the second-generation hybrid capture microplate test, comparing two different cervical specimen collection methods. Clin Diagn Virol. 1998 May 1; 10(1):49-56.

11. Scully RE, Bonfiglio TA, Kurman RJ, Silverberg SG, Wilkins EJ. Histological typing of female genital tract tumors. World Health Organization - International histological classification of tumors. 2nd ed. Berlin: Springler-Verlag; 1994.

12. Remmele W, Schicketanz KH. Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs subjective grading (IRS). Pathol Res Pract 1993;8:227-45.

13. R Development Core Team (2004). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3, URL http://www.R-project.org.

14. Ryu HS, Chang KH, Yang HW, Kim MS, Kwon HC, Oh KS. High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. Gynecol Oncol 2000; 76:320-5.

15. Kulkarni S, Rader JS, Zhang F, et al. Cyclooxygense-2 is overexpressed in human cervical cancer. Clin Cancer Res 2001; 7:429-34.

16. Dong YL, Gangula PR, Fang L, Yallampalli C. Differential expression of cyclooxygenase-1 and -2proteins in rat uterus and cervix during the estrous cycle, pregnancy, labor and in myometrial cells. Prostaglandins 1996; 52:13-34.

17. Shiozawa T, Shiohara S, Kanai M, Konishi I, Fujii S, Nikaido T. Expression of the cell cycle regulator p27 (Kip1) in normal squamous epithelium, cervical intraepithelial neoplasia, and invasive squamous cell carcinoma of the uterine cervix. Immunohistochemistry and functional aspects of p27 (Kip1). Cancer 2001; 92:3005-11.

18. Dai Y, Zhang X, Peng Y, Wang Z. The expression of cyclooxygenase-2, VEGF and PGs in CIN and cervical carcinoma. Gynecol Oncol 2005; 97:96-103.

19. Subbaramiah K, Zakim D, Weksler BB, Murata H. Inhibition of cyclooxygenase: a novel approach to cancer prevention. Proc Soc Exp Biol Med 1997; 216:201-10.

20. Sales KJ, Katz AA, Davis M, et al. Cyclooxygenase-2 expression and prostaglandin E_2 synthesis are up-regulated in carcinomas of the cervix: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. J Clin Endocrinol Metab 2001; 86:2243-9.

21. Lim HY, Joo HJ, Choi JH, et al. Increased expression of cyclooxygenase-2 protein in human gastric carcinoma. Clin Cancer Res 2000; 6:519-25.

Table 1 – Expression of COX-2 and Ki67 in CIN and cervical cancer

Histological grade	COX-2 expression			Ki67 expression	
	Negative/ Weak	Moderate/Strong	OR (95%CI)	Mean %	P*
Normal/ Cervicitis	6 (6.1%)	3 (4.5%)	Ref	20.4	Ref
CIN 1	20 (20.3%)	13 (19.3%)	0.8 (0.2 to 3.7)	23.6	0.66
CIN 2	7 (7.1%)	14 (20.9%)	0.2 (0.1 to 1.2)	33.8	0.13
CIN 3	62 (63.2%)	33 (49.3%)	0.8 (0.2 to 3.6)	45.5	0.001
Cancer	3 (3.1%)	4 (6.0%)	0.3 (0.1 to 2.5)	41.8	0.06
Total	98 (100)	67 (100)			

* adjusted for HPV detection

Table 2 - COX-2 expression as related to HPV infection and Ki67 expression

COX-2 expression	HPV			Ki67* expression	
	Positive	Negative	OR (95%CI)**	Mean %	P**
Negative/weak	54 (39.1%)	13 (48.1%)	Reference	37.6%	Reference
Moderate/strong	84 (60.9%)	14 (51.9%)	1.5 (0.6 to 4.2)	39.0%	0.87
TOTAL	138 (100)	27 (100)			

*Percentage of epithelial cells expressing Ki67

**Adjusted for CIN grade

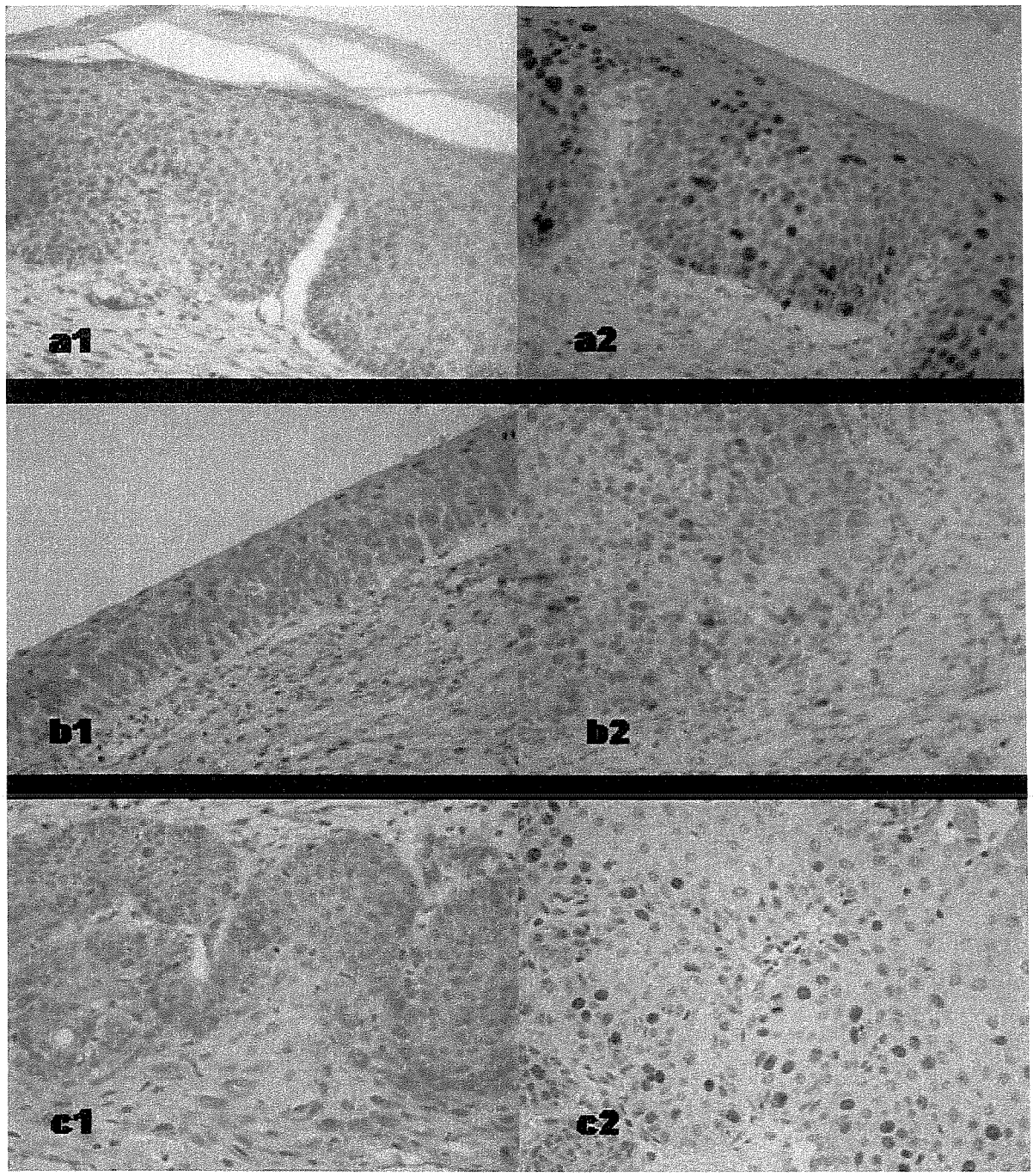


Figure 1 – Expression of COX-2 and Ki67 in selected CIN3 cases

Case A: 1) negative/weak COX-2 expression; 2): Ki67 >50% of the cells

Case B: 1) moderate COX-2 expression; 2) Ki67 >50% of the cells

Case C: 1) moderate/strong COX-2 expression; 2) Ki67 10-50% of the cells

3.2. Artigo 2

HPV persistence, expression of cyclooxygenase 2 (COX-2) and Ki67 protein as related to the risk of cervical intraepithelial neoplasia recurrence after diathermic conization

Luis Otavio Sarian[1]
Sophie Françoise Derchain[1]
Adriana Yoshida[1]
José Vassalo[2]
Francisco Pignataro[2]
Liliana Aparecida Lucci De Angelo Andrade[2]

1. Department of Obstetrics and Gynecology; 2. Department of Pathology, Universidade Estadual de Campinas (UNICAMP)

Grant: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processes number 00/06394-0 and 04/09309-5 and Conselho de Desenvolvimento Científico e Tecnológico (CNPq), process number 307252/2004-3

Corresponding author: Sophie F. Derchain, MD, PhD
Rua Antônio Hossri, 629 – Cidade Universitária
13083-370 – Campinas – SP.
Fone: +55-19-37889305 / Fax: + 55-19-3289 5935
E-mail: derchain@supernet.com.br

Running headline: COX-2 and Ki67 as related to recurrence after conization

Keywords: immunohistochemistry, MIB-1, treatment, cervical cancer

Summary

Objective: To assess the rate of persistence/recurrence of cervical intraepithelial neoplasia (CIN) following loop electrosurgical excision procedure (LEEP) as related to the cytoplasmic expression of cyclooxygenase-2 (COX-2), nuclear expression of Ki67 and high-risk Human papillomavirus (HPV) detection in up to 36 months follow-up. **Methods:** This is a prospective report on 104 non-consecutive women who attended consultations at the colposcopy clinics at the Medical School Hospital of the *Universidade Estadual de Campinas* (UNICAMP), due to abnormal Pap smears and that were treated by LEEP between February 2001 and April 2004. Patients were scheduled for follow-up visits at 6, 12, 18, 24 and 36 months after treatment, until September 2005. **Results:** Fourteen persistent/recurrent cases have been detected during follow-up. Survival analysis did not disclose any significant difference concerning cytoplasmic COX-2 protein expression or nuclear Ki67 expression and disease-free survival. Recurrent/persistent CIN was related only to HPV infection during follow-up (Risk Ratio 7.6; 95% confidence interval 2.1-28.6. **Conclusion:** In the present series, no differences in disease-free survival were detected in relation to cytoplasmic COX-2 protein expression and lesion growth rate.

Introduction

Recurrence rates following loop electrosurgical excision procedure (LEEP) for the treatment of cervical intraepithelial neoplasia (CIN) ranges from 5 to 10% [1] and residual disease can be found in 20-35% of hysterectomy specimens after this treatment [2,3]. Reports on disease outcome with complete and incomplete excision have shown that CIN may be cured even in the presence of residual disease [4], although patients with histologically-proven complete CIN removal may recur [5]. These figures emphasize the importance of close surveillance after cone treatment to ensure an early detection of residual disease or recurrence. This follow-up is traditionally performed with repeated colposcopic examination and cervical cytology (Pap smear).

With the confirmation of the causal role of Human papillomavirus (HPV) infection in the pathogenesis of CIN [6,7] and invasive cervical cancer, associated to the fact that the Pap test may be normal even in the presence of biopsy-confirmed high-grade CIN [1,8] an increased interest has been focused on HPV detection in the post-conization follow-up. Indeed, residual or recurrent disease only occurs in women with persistent HPV infection after treatment [9-13]. However, the potential of high-oncogenic HPV types to induce carcinogenesis is certainly incomplete, because only a small percentage (roughly 1%) of infected women will develop CIN and only 16% of women with CIN1 or CIN2, with confirmed HPV infection, will progress to CIN3 or cancer [14]. Therefore, several other factors should, at least in theory, play a role in the process that leads to recurrences.

The rate-limiting enzyme involved in the conversion of arachidonic acid to prostanglandins in inflammatory processes, Cyclooxygenase-2 (COX-2), has

also been shown to have a relation to carcinogenesis in humans. It is suggested that COX-2, besides its participation in inflammatory responses, might induce tumor proliferation and spread by enhancing mitogenesis, and by reducing cellular adhesion and the immune surveillance [15,16]. In cervical cancer, comparative studies found that COX-2 might be prognostic, correlating to a diminished response, in terms of survival, to radiotherapy and augmented resistance to chemotherapy [17, 18]. Currently under investigation, however, the expression of COX-2 in pre-invasive cervical lesions, or CIN, is not well understood. In a previous study, we reported on a series of 165 specimens of cervical lesions at various grades (ranging from cervicitis to cancer), but no relation of the protein expression with disease grade has been detected. Because of the scarcity of available data in the literature with respect to the prognostic value of COX-2 after CIN treatment with diathermic conization, we decided to undertake this prospective study, aimed at assessing the rate of persistent and recurrent CIN following LEEP as related to the cytoplasmic expression of COX-2, nuclear expression of Ki67 and HPV detection during follow-up.

Patients and methods

Patients selection and study design

The sample in this cohort study comprises 104 women, who were referred to the colposcopy clinics at the Medical School Hospital of the *Universidade Estadual de Campinas* (UNICAMP) due abnormal Pap smear and who were treated by LEEP between February 2001 and April 2004. Patients were scheduled for

follow-up visits at 6, 12, 18, 24 and 36 months after treatment, until September 2005, depending on their follow-up time. Inclusion criteria were CIN confirmed in the conization specimens, and the patient attending at least one follow-up visit. Exclusion criteria consisted of pregnancy, clinical signs of immunossuppression, and testing HIV positive. The study protocol was approved by the local Ethics Committee and all patients signed the informed consent.

Mean follow-up time was 17.5 months (range 6-36) and the study sample achieved a total of 1818 months/patient of follow-up. Patients missing in one of the routine appointments were contacted by phone or letter, when possible, and had a second appointment set to the nearest vacancy.

Consultation routine

At the pre-treatment visit, all women were submitted to an interview regarding clinical, social and demographic data. After being interviewed, a complete gynecological examination was performed, with collection of endocervical specimens for cervical cytology and high-risk HPV Hybrid Capture II (HCII) specimens followed by pelvic examinations, whuch were completed with colposcopy. Electrosurgical procedure was performed with high frequencies electrical generator (WEM, model SS-200) set at 50-60W cut and 50W coagulation, at blend mode. After cone resection, electrical coagulation (to stop bleeding) was performed with 50W. Epoxy-protected speculums were used for electrical insulation. Local anesthesia was introduced with 2% lidocaine plus nor-epinephrine 1/50.000, inoculated at 3, 6, 9 and 12o'clock in the cervix with gingival needles, 27G.

Dosage for each quadrant was about 0.4ml of anaesthetic plus nor-epinephrine. Smoke was evacuated with WEM Wavewac.

Follow-up (FU) visits included patient interview and complete gynecological examination, with collection of high-risk HC II at 6, 12, 24 and 36 months visit, Pap test samples and colposcopy at each visit. Patients presenting with abnormal Pap smear result or abnormal pattern on colposcopy underwent a new excision procedure, biopsy, LEEP, cold-knife conization, or hysterectomy.

HPV detection

The specimens for HC II were tested with probe B (high-risk HPVs: types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) [20] and the tests have been classified at the relative light unit/positive control (RLU/PC) ratio (RLU of specimen/mean RLU of two positive controls) of 1pg/ml or greater. These RLU/PC ratios provided an estimate of the amount of HPV-DNA in the specimens, i.e. the viral load in the sample. During FU, patients were regarded as having *persistent* HPV infections when the virus was detected in at least one FU visit. The storage of the specimens and all reagents as well as conduction of the tests took place at the Campinas State University Medical School Hospital Laboratory, following the manufacturer's instructions (*Digene Diagnostics Inc., USA*).

Histology

Histological samples consisted of LEEP specimens at baseline and biopsy, conization or hysterectomy specimens during follow-up, which were fixed in 10% phosphate buffered formalin, embedded in paraffin, and stained with hematoxylin

and eosin (H&E). Biopsies were analyzed according to the World Health Organization criteria's [21] and classified as normal/cervicitis, CIN1, CIN2, CIN3, invasive squamous cell carcinoma.

Immunohistochemistry (IHC)

IHC reactions were performed on slides obtained from the tissue blocks that contained the most significant lesion detected on H&E assessment. Five micrometer sections were obtained from the tissue blocks and transferred to silanized glass slides and dried overnight at 37° C. The sections were deparaffinized using a sequence of xylene, followed by a washes in graded ethanol and phosphate buffered saline (PBS; pH7.4). Pretreatment of the slides consisted of microwaving for 15 min in 10mmol/L sodium citrate (pH 7.0). After washing the slides with PBS, endogenous peroxidase was blocked by 3% hydrogen peroxide (H_2O_2), by three sequential incubation baths, 3 min each. Samples were then incubated for 30 min in a humid chamber at 37°C with the antibodies: anti-COX2 (1:100 dilution), Ki67 (1:150 dilution) (Ki67, Dako, A/S, Copenhagen, Denmark). Afterwards, slides were incubated overnight at 4°C. Slides were then incubated with biotinylated linked secondary antibody (Dako) for 30 min and with a solution of avidin biotinylated peroxidase. For slides development a diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate was used, for 6 minutes at 37°C. The slides were washed with water and counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted with resin Entellan®. The corresponding sections without the primary antibody were employed as negative controls.

Assessment of IHC slides

Reading of all slides was performed by one investigator, who was blinded to the H&E diagnosis rendered by a pathologist and to the HPV status of the patients. Digitalized photographs of high power fields (400x) were taken from hot-spots of reaction with a Nikon COOLPIX Camera 995, and then images were ported to Personal-Computer based software for histological analyses (Imagelab 2000).

COX-2 immonoreactivity grading was based on the German ImmonoReactive score [12]. Firstly, staining intensity was rated on a scale from 0 to 3, with 0 being no staining at all, 1 weak staining, 2 moderate and 3 strong staining. Then, positive and negative cells were counted, with no less than 500 cells as the minimum acceptable count number. The percentage of positive cells was then scored as: no staining as 0; 1-10% as 1; 11 to 50% as 2; 51 to 80% as 3 and 81% to 100% as 4. The final score was calculated by multiplying the score obtained with the staining intensity by that derived from the percentage of positive cells, achieving theoretical results ranging 0 to 12. A final score of 0 was regarded as negative, 1-4 as weak, 5-8 as moderate and 9 to 12 was considered as strong immunoreactivity. For statistical purposes, two groups were formed: negative and weak immunoreactivity; moderate and strong immunoreactivity. Slides with colitis known to be positive for the COX-2 were used as positive controls.

Ki67 nuclear expression

A continuum percentage of cells expressing Ki67 in their nuclei was used for the assessment of cell proliferation rate. A minimum count of 500 nuclei was considered mandatory. Nuclear staining of basal cells was considered as the

internal positive control of reaction. Specimens of lung adenocarcinoma, known to be strongly positive for Ki67, were used as external reaction positive control.

Statistical analysis

A regression model, based upon the Kaplan-Meyer survival analysis, has been fit in order to evaluate the possible relations of disease-free survival to CIN grade, COX-2 and Ki67 expression, margin status and the detection of HPV during follow-up with. Risk ratios (RR) for each variable under evaluation were calculated with the exponentiation of Cox proportional hazards coefficients, derived from the survival model. Disease-free survival curves were obtained for patients with zero/weak and moderate/strong COX-2 protein expression. Data were recorded in an OpenOffice® spreadsheet and statistical treatment has been performed with the R *Environment* [23] statistical software package, set to 95% confidence intervals (95%CI).

Results

Patients' ages ranged 21.8-61.6 years (mean 35.9 years; data not shown). Fourteen persistent/recurrent cases have been detected during follow-up. Of the persistent/recurrent cases, 11 had moderate to strong COX-2 protein expressing lesions at baseline assessment; none had specimens with weak (0-10%) Ki67 expression at baseline, being that five expressed the antigen in 11-50% of the cells and nine in more than 50% of the cells. Involved margins at baseline histological assessment were present in 5 of the 14 persistent/recurrent cases, but 18 (78%) out of the 23 women with involved margins did not had

persistent/recurrent disese diagnosed throughout FU. During FU, 11 out of the 14 cases of persistent/recurrent disease were diagnosed with persistent HPV infections, contrasting to 24/90 women without disease. Of the variables under scrutiny, persistent/recurrent was related only to HPV infection during FU (OR 7.6; 95%CI 2.1-28.6), as displayed in **Table 1.** Survival analysis did not disclose any significant difference concerning COX-2 protein expression and disease-free survival (p=0.19; **Figure 1**).

Discussion

The incomplete potential of HPV to elicit carcinogenesis calls for a in-depth study of other clinical and pathological characteristics that might play a role in the development of CIN and cancer. Several previous reports suggest the relation of COX-2 expression with carcinogenesis in a wide range of tumors, e.g esophagus, colon, stomach and breast [15, 24, 25]. In cervical cancer, COX-2 expression has been shown to correlate with increased tumor aggressiveness and metastasis in squamous cell carcinoma and adenocarcinoma of the cervix (26, 27, 28), and diminished disease response to radiotherapy as well. These biological features of the marker prompted investigators to evaluate whether COX-2 expression could possibly act as prognostic factor for recurrences following conservative CIN treatment [19]. Such a marker would help in distinguishing women at increased risk for disease recurrence, therefore allowing practitioners to tailor differentiated follow-up strategies.

In the present series, no differences in disease-free survival were detected in relation to cytoplasmatic COX-2 expression. Contrasting to the present findings,

a recent retrospective report on 62 women, followed-up for one year, detected a borderline tendency of increased COX-2 expression in specimens of women that had a recurrence [19]. To our knowledge, there is no further data on the subject available in current literature. Therefore, it is to be anticipated that the present report is certainly the largest cohort of CIN-treated to be prospectively evaluated in regards to the COX-2 expression and disease recurrence to date.

Recently, COX-2 cytoplasmic expression has been suggested to directly correlate with CIN grade, but at borderline significance (p=0.06;) [19]. The enzyme expression has been shown to correlate well with levels of the cyclin-dependent kinase inhibitor p27, whose levels, contrary to what happened with COX-2, decreased as disease progressed [19]. Because p27 is important in regulating the G1/S transition, its reverse association with COX-2 expression suggests that this enzyme levels are much likely to be affected by the disruptive mechanisms of the cell cycle that lead to uncontrollable proliferation in neoplasia. Most unfortunately, however, the expression of COX-2 was not clearly related to persistence or recurrence of the disease in up to 36 months in the present study.

Testing for HPV at baseline and during follow-up enabled the assessment of CIN behavior, clinically and histologically, as related to the viral infection. As commented on before, there was no association between HPV status, at baseline or during follow-up, with the cytoplasmic expression of COX-2. Nevertheless, the prevalence of the virus is very high at enrollment, obviously as a consequence of the selection criteria adopted. The lower HPV detection rates were found among patients with CIN1, which can be explained a) by the nature of the lesion, sometimes related to viral types not included in the probe HCII assay pools and b) by

the cumbersome evaluation of mild cervical dysplasia, as previously debated on the ALTS studies[14]. Concerning HPV during follow-up, the present results are in perfect alignment with previous cohorts, in which persistence/recurrence rates were remarkably more elevated among patients with persistence of the viral infection during follow-up, regardless of the technique implemented to assess the viral status of the host.

The discriminating potential of a biological marker, to detect women at increased risk of CIN recurrences following conservative treatments, should be regarded from the individual (patient and her physician) and public-health (planners) standpoints. For the patient and her physician, it is certainly of major importance to know the odds of having a recurrence in the short or medium-term, because cervical neoplasia has the potential to affect fertility, self-image and eventual follow-up consultations and treatments might take several working hours. From the public-health standpoint, it is necessary to consider that, severely affected by the lack of human and economic resources, the underprivileged regions of the globe in which prevalence of cervical disease is higher struggle to construct an infrastructure for the precocious diagnose and treatment. In this context, an affordable technique that could help distinguishing women at increased risk of CIN recurrence would be extremely welcomed. Immunohistochemistry certainly has the prerequisite of affordability, but no antigen has been proven of prognostic relevance for the recurrence of CIN after conservative treatments. It is unfortunate that the present work possibly adds another biological element to the list of useless biomolecular markers in this regard.

References

1. Flannelly G, Bolger B, Fawzi H, De Lopes AB, Monaghan JM. Follow up after LLETZ: could schedules be modified according to risk of recurrence? BJOG 2001; 108:1025-30.
2. Lin CT, Tseng CJ, Lai CH, Hsueh S, Huang KG, Huang HJ, Chao A. Value of human papillomavirus deoxyribonucleic acid testing after conization in the prediction of residual disease in the subsequent hysterectomy specimen. Am J Obstet Gynecol 2001;184:940-5.
3. Lu CH, Liu FS, Tseng JJ, Ho ES. Predictive factors for residual disease in subsequent hysterectomy following conization for CIN III. Gynecol Oncol 2000; 79:284-8.
4. Paraskevaidis E, Kalantaridou SN, Paschopoulos M, Zikopoulos K, Diakomanolis E, Dalkalitsis N, Makrydimas G, Pappa L, Malamou-Mitsi V, Agnantis NJ. Factors affecting outcome after incomplete excision of cervical intraepithelial neoplasia. Eur J Gynaecol Oncol 2003; 24:541-3.
5. Milojkovic M. Residual and recurrent lesions after conization for cervical intraepithelial neoplasia grade 3. Int J Gynaecol Obstet 2002; 76:49-53.
6. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999; 189(1):12-9.
7. Wacholder S. Chapter 18: Statistical issues in the design and analysis of studies of human papillomavirus and cervical neoplasia. J Natl Cancer Inst Monogr 2003; 31:125-30.

8. Tangtrakul S, Linasmita V, Israngura N, Srisupundit S, Bullangpoti S, Wilailak S. Detection of residual disease by cytology in patients with cervical intraepithelial neoplasia III post-large loop excision of the transformation zone. J Obstet Gynaecol Res 2002; 28:95-8.
9. Chua KL, Hjerpe A. Human papillomavirus analysis as a prognostic marker following conization of the cervix uteri. Gynecol Oncol 1997; 66:108-13.
10. Bollen LJ, Tjong-A-Hung SP, van der Velden J, Mol BW, ten Kate FW, ter Schegget J, Bleker OP. Prediction of recurrent and residual cervical dysplasia by human papillomavirus detection among patients with abnormal cytology. Gynecol Oncol 1999; 72:199-201.
11. Nagai Y, Maehama T, Asato T, Kanazawa K. Persistence of human papillomavirus infection after therapeutic conization for CIN 3: is it an alarm for disease recurrence? Gynecol Oncol 2000; 79:294-9.
12. Nobbenhuis MA, Meijer CJ, van den Brule AJ, Rozendaal L, Voorhorst FJ, Risse EK, Verheijen RH, Helmerhorst TJ. Addition of high-risk HPV testing improves the current guidelines on follow-up after treatment for cervical intraepithelial neoplasia. Br J Cancer 2001 23; 84:796-801.
13. Zielinski GD, Rozendaal L, Voorhorst FJ, Berkhof J, Snijders PJ, Risse EJ, Runsink AP, de Schipper FA, Meijer CJ. HPV testing can reduce the number of follow-up visits in women treated for cervical intraepithelial neoplasia grade 3. Gynecol Oncol 2003; 91:67-73.
14. Schiffman M, Solomon D. Findings to date from the ASCUS-LSIL Triage Study (ALTS). Arch Pathol Lab Med 2003;127:946-9.

15. Shamma A, Yamamoto H, Doki Y, Okami J, Kondo M, Fujiwara Y, et al. Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. Clin Cancer Res 2000; 6:1229-38.

16. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999; 19:790816.

17. Gaffney DK, Holden J, Zempolich K, Murphy KJ, Dodson M. Elevated cyclooxygenase-2 expression correlates with diminished survival in carcinoma of the cervix treated with radiotherapy. Int J Radiat Oncol Biol Phys 2001; 49:1213-7.

18. Ferrandina G, Lauriola L, Distefano MG, Zannoni GF, Gessi M, Legge F, et al. Increased cyclooxygenase-2 expression is associated with chemotherapy resistance and poor survival in cervical cancer patients. J Clin Oncol 2002; 20:973-81.

19. Farley J, Uyehara C, Hashiro G, Belmap C, Birrer M, Salminen E. Cyclooxygenase-2 expression predicts recurrence of cervical dysplasia following loop electrosurgical excision procedure. Gynecol Oncol 2004; 92: 596-602

20. Nindl I, Lorincz A, Mielzynska I et al. Human papillomavirus detection in cervical intraepithelial neoplasia by the second-generation hybrid capture microplate test, comparing two different cervical specimen collection methods. Clin Diagn Virol 1998 May 1; 10(1):49-56.

21. Scully RE, Bonfiglio TA, Kurman RJ, Silverberg SG, Wilkins EJ. Histological typing of female genital tract tumors. World Health Organization - International histological classification of tumors. 2nd ed. Berlin: Springler-Verlag; 1994.

22. Remmele W, Schicketanz KH. Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs subjective grading (IRS). Pathol Res Pract 1993;8:227-45.

23. R Development Core Team (2004). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3, URL http://www.R-project.org.

24. Lim HY, Joo HJ, Choi JH, Yi JW, Yang MS, Cho DY, et al. Incresed expression of cyclooxygenase-2 protein in human gastric carcinoma. Clin Cancer Res 2000; 6:1229-38.

25. Hwang D, Scollard D, Byrne J, Levine E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J Natl Cancer Inst 1998; 90:455-60.

26. Ferrandina G, Ranelletti FO, Legge F, Gessi M, Salutari V, Distefano MG, et al. Prognostic role of the ratio between cyclooxygenase-2 in tumor and stroma compartments in cervical cancer. Clin Cancer Res 2004; 10:3117-3123.

27. Kim MH, Seo SS, Song YS, Kang DH, Park IA, Kang SB, Lee HP, et al. Expression of cyclooxygenase-1 and cyclooxygenase-2 associated with expression of VEGF in primary cervical cancer and at metastatic lymph nodes. Gynecol Oncol 2003; 90: 83-90.

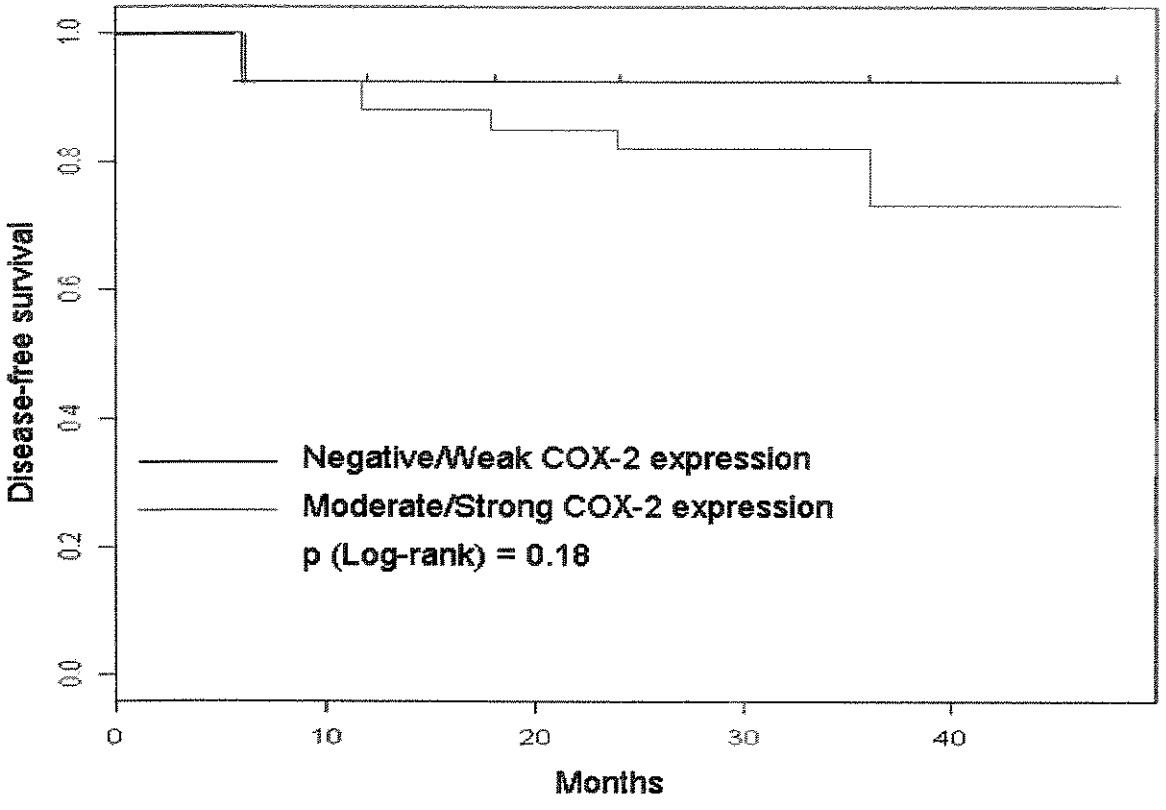
28. Ryu HS, Chang KH, Yang HW, Kim MS, Kwon HC, Oh KS. High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. Gynecol Oncol 2000; 76:320-325.

Table 1 – CIN persistence/recurrences as related to COX-2 and Ki67 expression, HPV persistence during follow-up and margin status

Characteristic	Persistence/recurrence Yes N (%)	No N (%)	Total N (%)	RR* (95%CI)
CIN grade				
Low	2 (11)	16 (89)	18 (100)	Ref
High	12 (14)	74 (86)	86 (100)	0.7 (0.1 to 4.6)
COX-2				
Zero/weak	3 (7)	37 (93)	40 (100)	Ref
Mod/strong	11 (17)	53 (83)	64 (100)	1.7 (0.4 to 6.7)
Ki67 (%)				
0-10%	0 (0)	9 (0)	9 (100)	
11-50%	5 (9)	51 (91)	56 (100)	Ref
>50%	9 (22)	30 (78)	39 (100)	2.9 (0.8 to 11.0)
Margins				
Free	9 (11)	72 (89)	81 (100)	Ref
Involved	5 (22)	18 (78)	23 (100)	7.6 (2.1 to 28.6)
HPV during FU**				
Non-persistent	3 (4)	66 (96)	69 (100)	Ref
Persistent	11 (31)	24 (69)	35 (100)	2.5 (0.8 to 8.1)

*Risk Ratios calculated for the overall recurrence/persistence rates. Not related to the moment at which disease was detected **Follow-up.

Figure 1 – Disease-free survival of CIN treated patients in relation to cytoplasmic COX-2 protein expression



4. Discussão

Marcadores biomoleculares têm sido objeto de estudo em praticamente todos os tipos de tumores que acometem os humanos. Vários destes marcadores encontraram relevante aplicação na clínica, e alguns exemplos estão ligados à prática ginecológica: receptores de estrógeno e progesterona, C-ERB-B2, Ki67 nas neoplasias mamárias, vimentina, antígeno cárcino-embrionário e receptores estrogênicos nas neoplasias do endométrio, entre outros. Para que se pudesse chegar aos marcadores com efetiva repercussão clínica, certamente uma ampla variedade de candidatos foi testada, e a maioria deles, descartada.

Recentemente, McCluggage (2004) publicou extensa revisão sobre o valor da imuno-histoquímica no diagnóstico de neoplasias uterinas. Na introdução, o autor comenta que "[...] existe grande quantidade de publicações sobre o valor da imunohistoquímica em relação a várias neoplasias [...] o que geralmente resulta em patologistas assumindo que a técnica pode fornecer um diagnóstico final, o que é claramente falso. Embora a imunohistoquímica possa ser de grande valor, se os

seus resultados forem interpretados sem o devido conhecimento, grandes erros diagnósticos poderão ser cometidos".

A presente tese procurou tratar da expressão, em lesões escamosas do colo do útero, de dois marcadores biomoleculares, a COX-2 e o Ki67, os quais têm recebido grande atenção na literatura recente, em diversas neoplasias. O conhecimento obtido, nas últimas décadas, sobre o incompleto potencial oncogênico do HPV foi uma das motivações para a realização do presente estudo. Embora seja verdade que apenas mulheres infectadas pelo vírus desenvolvem NIC e câncer do colo, somente uma pequena parte daquelas portadoras do HPV virá a ter a neoplasia. Nas mulheres tratadas da NIC através de técnicas que permitem conservar o colo do útero (conização com bisturi ou com alça diatérmica), embora o vírus persista em 25% a 30% delas, apenas 10% a 15% terão recidivas (Sarian et al., 2004). Dados acumulados a respeito da carcinogênese nas lesões escamosas do colo do útero permitiram determinar uma série de marcadores biológicos que potencialmente teriam algum valor preditivo ou prognóstico em relação às recidivas das lesões precursoras. Nesse ambiente ainda se mantinha muito pouco estudada a participação da COX-2, enzima comprovadamente relevante nos processos inflamatórios e na gênese de vários tumores - entre eles o câncer do colo - no potencial de recidivas das lesões pré-invasoras.

Optou-se por dividir o estudo em dois artigos. O primeiro teve a finalidade de relatar os achados sobre a expressão da COX-2 e suas relações com as características das lesões, como sua gravidade e taxa de proliferação celular, bem como em relação à detecção do HPV de alto risco oncogênico. Nesse

artigo foram incluídas 165 mulheres, em cujos espécimes de conização havia sido encontrado cervicite, NIC ou câncer. Esta abordagem permitiu estabelecer comparações sobre a expressão da COX-2 em tecidos com e sem neoplasia, o que possibilitou verificar que não havia diferença em relação à expressão citoplasmática de COX-2 em tecidos com características inflamatórias, quando comparados àqueles com NIC ou câncer. Este achado corrobora os de um estudo prévio, desenvolvido recentemente na China (Dai et al., 2005), em que a expressão intranuclear de mRNA da COX-2 foi estatisticamente semelhante em tecidos com inflamação (cervicite), quando comparados àqueles com NIC. Todavia, os presentes achados não reproduzem os que Farley et al. (2004) relataram, ou seja, que a expressão da COX-2 eleva-se em paralelo ao aumento da gravidade das lesões.

O segundo artigo tratou das possíveis associações entre a expressão de COX-2 e o comportamento das recidivas de NIC. Nesta publicação procurou-se incluir um fator de grande interesse na análise dos riscos relativos à expressão da COX-2: a detecção do HPV no seguimento. Também foi incluída a análise das margens dos espécimes, o que pode estar relacionado com a persistência da NIC. Entretanto, não houve associação do estado das margens com a freqüência geral de recidivas. Das variáveis estudadas, entre elas a expressão da COX-2 e taxa de proliferação celular, nenhuma esteve significativamente associada às recidivas, exceto a persistência do HPV.

A COX-2, como mencionado anteriormente, vem sendo objeto de interesse de diversos autores, e várias correlações positivas entre a expressão deste antígeno e o comportamento biológico e clínico de diversos tumores, entre os quais o

câncer do colo, têm sido demonstradas. Ao se avaliar a expressão da COX-2 em diferentes graus da NIC e sua relação com as recidivas, descartando associações significativas em ambos os casos, o presente estudo fornece um importante elemento para discussão em relação ao comportamento das lesões escamosas pré-invasoras do colo do útero. Pode-se antecipar que o intenso componente inflamatório na cérvice acometida pelo HPV desencadeia forte expressão da COX-2, independentemente do grau da lesão, impedindo qualquer associação deste marcador com fatores histológicos ou clínicos, antes que a lesão torne-se invasora.

5. Conclusões

- **Artigo 1**

A expressão citoplasmática da COX-2 não esteve relacionada com a gravidade da lesão cervical, com a infecção pelo HPV e com a expressão do Ki67. Esta última, entretanto, esteve significativamente associada à gravidade da lesão.

- **Artigo 2**

A expressão citoplasmática de COX-2 não foi um fator prognóstico para a recorrência da NIC após conização com alça diatérmica. Entre os fatores estudados (expressão da COX-2 e do Ki67, margens de conização e infecção persistente pelo HPV), apenas a infecção pelo HPV durante o seguimento esteve relacionada com a recidiva da NIC.

6. Referências Bibliográficas

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell Published by Garland Publishing, 1994 Chapter 17 – The General Startegy of the Cell Cycle. Disponível online em: http://www.ncbi.nlm.nih.gov/entrez/books/cell.section4584

Costa S, De Simone P, Venturoli S, Cricca M, Zerbini ML, Musiani M, et al. Factors predicting human papillomavirus clearance in cervical intraepithelial neoplasia lesions treated by conization. ***Gynecol Oncol*** 2003; 90:358-65.

Dai Y, Zhang X, Peng Y, Wang Z. The expression of cyclooxygenase-2, VEGF and PGs in CIN and cervical carcinoma. ***Gynecol Oncol*** 2005; 97:96-103.

DuBois RN, Giardiello FM, Smalley WE. Non steroidal antiinflamatory drugs, eicosanoids and colorrectal cancer. ***Gastroenterol Clin North Am*** 1996; 25:773-91.

Duggan BD, Felix JC, Mudespah LI, Gebhardt JA, Groshen S, Morrow CP. Cold-knife conization versus conization by the loop eletrosurgical excision procedure: a randomized, prospective study. ***Am J Obstet Gynecol*** 1999; 180:276-82.

Farley J, Uyehara C, Hashiro G, Belnap C, Birrer M, Salminen E. Cyclooxygenase-2 expression predicts recurrence of cervical dysplasia following loop electrosurgical excision procedure. ***Gynecol Oncol*** 2004; 92:596-602.

Flannelly G, Langhan H, Jandial L, Mana E, Campbell M, Kitchener H. A study of treatment failures following large loop excision of the transformation zone for the treatment of cervical intraepithelial neoplasia. ***Br J Obstet Gynaecol*** 1997; 104:718-22.

Flannelly G, Bolger B, Fawzi H, De Lopes AB, Monaghan JM. Follow up after LLETZ: could schedules be modified according to risk of recurrence? ***BJOG*** 2001; 108:1025-30.

Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D, et al. Ki67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papillomavirus-related cervical neoplasia. ***Am J Surg Pathol*** 2001; 25:884-91.

Kruse AJ, Baak JP, de Bruin PC, Jiwa M, Snijders WP, Boodt PJ, et al. The HS . Ki67 immunoquantitation in cervical intraepithelial neoplasia (CIN): a sensitive marker for grading. ***J Pathol*** 2001; 193:48-54.

Kulkarni S, Rader JS, Zhang F, Liapis H, Koki AT, Masferrer JL, et al. Cyclooxygenase-2 is overexpressed in human cervical cancer. ***Clin Cancer Res*** 2001; 7:429-34.

Lim HY, Joo HJ, Choi JH, Yi JW, Yang MS, Cho DY, et al. Increased expression of cyclooxygenase-2 protein in human gastric carcinoma. ***Clin Cancer Res*** 2000; 6:519-25.

Lin CT, Tseng CJ, Lai CH, Hsueh S, Huang KG, Huang HJ, et al. Value of human papillomavirus deoxyribonucleic acid testing after conization in the prediction of residual disease in the subsequent hysterectomy specimen. ***Am J Obstet Gynecol***. 2001;184:940-5.

Nagai Y, Maehama T, Asato T, Kanazawa K. Persistence of human papillomavirus infection after therapeutic conization for CIN 3: is it an alarm for disease recurrence? ***Gynecol Oncol*** 2000; 79:294-9.

Nobbenhuis MA, Meijer CJ, van den Brule AJ, Rozendaal L, Voorhorst FJ, Risse EK, et al. Addition of high-risk HPV testing improves the current guidelines on follow-up after treatment for cervical intraepithelial neoplasia. ***Br J Cancer*** 2001; 84:796-801.

Norrish AE, Jackson RT, McRae CU. Non-steroidal anti-inflammatory drugs and prostate cancer progression. ***Int J Cancer*** 1998; 77:511-5.

Östor AG. The natural history of cervical intraepithelial neoplasia: a critical review. ***Int J Gynecol Pathol*** 1993; 12:186-92.

Paraskevaidis E, Kalantaridou SN, Paschopoulos M, Zikopoulos K, Diakomanolis E, Dalkalitsis N, et al. Factors affecting outcome after incomplete excision of cervical intraepithelial neoplasia. ***Eur J Gynecol Oncol*** 2003; 24:541-3.

Ryu HS, Chang KH, Yang HW, Kim MS, Kwon HC, Oh KS. High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. *Gynecol Oncol* 2000; 76:320-5.

Sarian LO, Derchain SF, Andrade LA, Tambascia J, Morais SS. HPV DNA test and Pap smear in detection of residual and recurrent disease following loop electrosurgical excision procedure (LEEP) of high-grade cervical intraepithelial neoplasia (CIN). ***Gynecol Oncol*** 2004; 94:181-6.

Shamma A, Yamamoto H, Doki Y, Okami J, Kondo M, Fujiwara Y. Up regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. ***Clin Cancer Res*** 2000; 6:1229-38.

Tangtrakul S, Linasmita V, Israngura N, Srisupundit S, Bullangpoti S, Wilailak S. Detection of residual disease by cytology in patients with cervical intraepithelial neoplasia III post-large loop excision of the transformation zone. ***J Obstet Gynaecol Res*** 2002; 28:95-8.

Tsujii M, Kawano S, Tsuji S. Cyclooxygenase-2 regulates angiogenesis induced by colon câncer cells. ***Cell*** 1998; 93:705-16.

Wacholder S. Chapter 18: Statistical issues in the design and analysis of studies of human papillomavirus and cervical neoplasia. J ***Natl Cancer Inst Monogr*** 2003; 31:125-30.

Wentzensen N, Vinokurova S, von Knebel Doeberitz M. Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. ***Cancer Res*** 2004; 64:3878-84.

Zielinski GD, Rozendaal L, Voorhorst FJ, Berkhof J, Snijders PJ, et al. HPV testing can reduce the number of follow-up visits in women treated for cervical intraepithelial neoplasia grade 3. ***Gynecol Oncol*** 2003; 91:67-73.

7. Bibliografia de Normatizações

FRANÇA, J.L.; BORGES, S.M.; VASCONCELLOS, A.C.; MAGALHÃES, M.H.A. – **Manual para normatização de publicações técnico-científicas**. 4ªed., Editora UFMG, Belo Horizonte, 1998. 213p.

Normas e procedimentos para publicação de dissertações e teses. Faculdade de Ciências Médicas, UNICAMP. Ed. SAD – Deliberação CCPG-001/98 (alterada 2005).

8. Anexos

8.1. Anexo 1 – Termo de Consentimento Livre e Esclarecido

PERSISTÊNCIA DO DNA-HPV APÓS A CONIZAÇÃO COM CIRURGIA DE ALTA FREQÜÊNCIA (CAF) EM MULHERES COM NIC 2 OU 3

Eu, Sra _____, portadora do RG_____, atendida no Centro de Atenção Integral à Saúde da Mulher (CAISM) no ambulatório de Patologia Cervical, participo da pesquisa "Persistência do DNA-HPV após a Conização com Cirurgia de Alta Freqüência (CAF) em mulheres com NIC 2 ou 3". Fui convidada a participar de uma continuação desta pesquisa que será realizada com o material que eu já coletei antes (aquela que identifica o vírus). Sei que este estudo tem como objetivo avaliar a presença do HPV de alto risco oncológico no meu colo uterino antes e após a conização por cirurgia de alta freqüência. O exame que foi feito antes identificou se tinha vírus ou não e este novo teste, chamado PCR irá determinar o tipo específico de vírus eventualmente presente no material que eu já coletei.

Fui esclarecida quanto ao meu direito de não participar da pesquisa e a não aceitação na participação no estudo não implicará na perda dos direitos iniciais rotineiramente oferecidos pelo ambulatório. Sei também que a qualquer momento posso desistir de participar da pesquisa, sem nenhum dano para a minha saúde e tratamento. Também fui

informada de que não terei custos para participar da pesquisa, pois, o intervalo entre as consultas será o mesmo realizado de rotina no ambulatório. Sei que todas as informações pessoais serão avaliadas somente pelo médico que me atendeu e que as fichas ficarão de posse do(s) Doutore(s) responsáveis pela pesquisa, Doutora Sophie Françoise Mauricette Derchain e Dr. Luis Otávio Zanatta Sarian, que manterão o sigilo da fonte destas informações. Em caso de dúvidas ou esclarecimento, tenho o direito de entrar em contato com os Drs. Luís Otávio Zanatta Sarian e Sophie F M Derchain, no telefone 3788-9305 ou com o Comitê de Ética em Pesquisa da UNICAMP no telefone 3788-8936.

Nome da paciente _____

Assinatura da paciente: _____

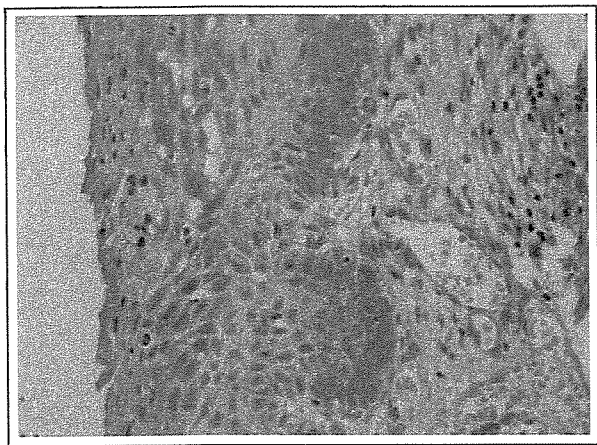
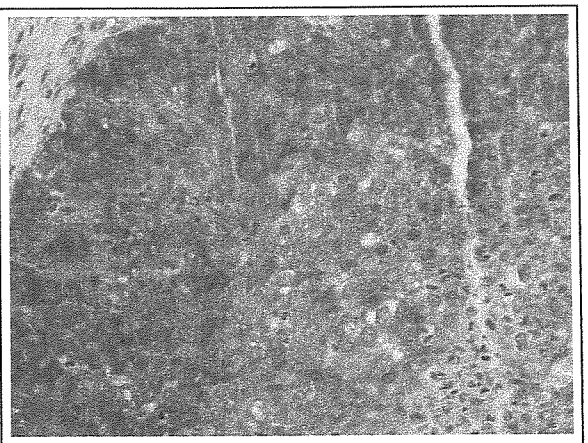
Campinas, _____ de _____ de 200_____.

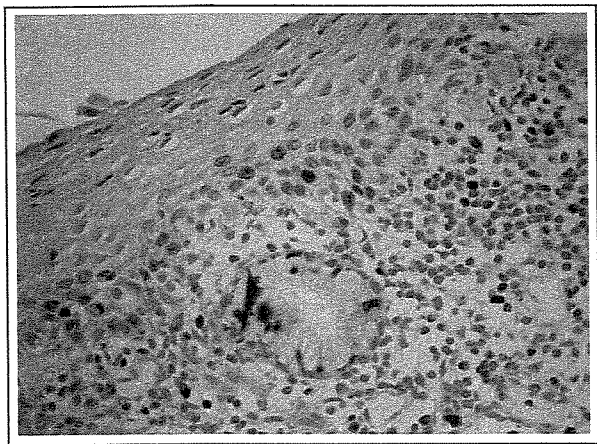
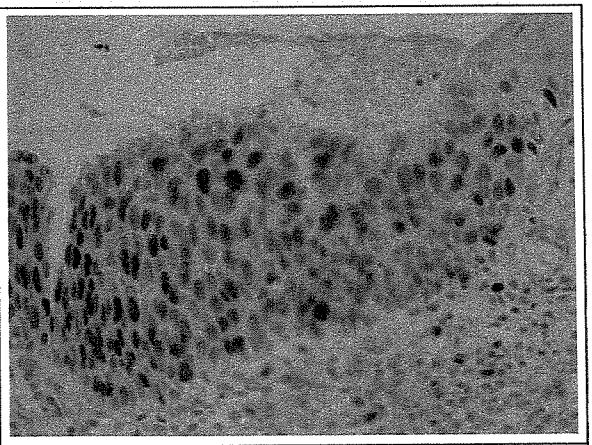
Nome do pesquisador _____

Assinatura do pesquisador: _____

Campinas, _____ de _____ de 200_____.

8.2. 8.2 Anexo 2 – Painel Imuno-histoquímico

	
COX-2 Intensidade: moderada Proporção de positivas: 10%-50% Expressão: Moderada	COX-2 Intensidade: forte Proporção de positivas: >80% Expressão: Forte

	
Ki67 Positivo em 34% das células	Ki67 Positivo em >90% das células