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SISTEMAS DE VISUALIZAÇÃO IMUNO-HISTOQUÍMICA LIVRES DE BIOTINA PARA AVALIAÇÃO DE RECEPTOR DE ESTRÓGENO EM CÂNCER DE MAMA: ANÁLISE EMPREGANDO DIGITALIZAÇÃO DE LÂMINAS, ACESSO REMOTO E PROGRAMA ANALISADOR DE IMAGENS

Belo Horizonte

Julho de 2009

UNIVERSIDADE FEDERAL DE MINAS GERAIS - FACULDADE DE MEDICINA

TESE DE DOUTORADO

SISTEMAS DE VISUALIZAÇÃO IMUNO-HISTOQUÍMICA LIVRES DE BIOTINA PARA AVALIAÇÃO DE RECEPTOR DE ESTRÓGENO EM CÂNCER DE MAMA: ANÁLISE EMPREGANDO DIGITALIZAÇÃO DE LÂMINAS, ACESSO REMOTO E PROGRAMA ANALISADOR DE IMAGENS

Tese apresentada ao curso de Pós-Graduação em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do Título de Doutor em Patologia

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Belo Horizonte

Julho de 2009

Este trabalho foi realizado no Laboratório de Patologia Mamária da Faculdade de Medicina da Universidade Federal de Minas Gerais, tendo a colaboração dos Deptos de Patologia do Hospital A.C. Camargo (Dr. Fernando Augusto Soares) e Faculdade de Ciências Médicas da Universidade Estadual de Campinas (Drs. José Vassallo e Natália Moraes Guimarães Schenka, e apoio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo À Pesquisa do Estado de Minas Gerais (FAPEMIG) e Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). Parte do trabalho foi realizado em Programa de Doutorado no Exterior (PDEE) desenvolvido no Laboratório University College London Advanced Diagnostics da Universidade de Londres (UCL), com bolsa da CAPES, tendo como mentor o Sr Keith Miller, diretor do United Kingdom National Quality Assessment Service (UK-NEQAS ICC & FISH) e chefe da imuno-histoquímica do laboratório de base deste serviço e da Universidade de Londres.

DEDICO

A todas as pacientes cuja carne foi motivo do nosso estudo

Aos meus pais Carlos e Ângela, às minhas irmãs Renata e Bruna, à minha filha Isabela e aos meus amigos pelo mundo

AGRADECIMENTOS

Agradeço à minha família, minha filha e aos meus amigos

Agradeço ao meu orientador estrangeiro e amigo Sr Keith Miller pelo conhecimento dispensado, ajuda material, financeira e amizade.

Agradeço ao Dr. Fernando Soares pela disponibilização de material para a realização do estudo, pelo convite de trabalho e, acima de tudo, pela confiança e amizade.

Agradeço ao Dr. José Vassallo por disponibilizar casos para o estudo e pela confiança, amizade e ensinamentos filosóficos diários.

Agradeço a Merdol, John Bartlett, Bharat Jasani, Ailin Rhodes, Jess, Gabie, Kzzy, Adriane, Phill, Josi, Crini, Uga, Elder, Bruno, Diogo, Marcos, Nicole e Anne pelos momentos em Londres.

Aos meus amigos Lucas Oliveira, Lucas Roque, Felipe Bittencourt, Marcio Carvalho e Augusto Horta.

Aos amigos do laboratório Gislene Rocha, Marina De Brot, Flávio Oliveira, Luciene Tafuri, Cristiana Nunes, Vanessa Marinho, Fernanda Sanches, Danielle e Vinicius.

À FAPEMIG, CNPq e CAPES pelas bolsas oferecidas desde a minha iniciação científica até a defesa deste doutorado com período sanduíche. Um total de sete anos de bolsa ininterruptos.

Agradeço à minha orientadora e amiga Helenice por tudo e principalmente por acreditar.

RESUMO

Introdução: uma nova geração de sistemas de visualização poliméricos livres de biotina (SPLB) para imuno-histoquímica têm sido desenvolvidos. **Objetivo:** comparamos os SPLB com os sistemas streptavidina-biotina (SABS) para a avaliação de imuno-reatividade para receptor de estrógeno (RE) em câncer de mama. **Material e método:** o anticorpo anti-receptor de estrógeno, clone SP1, foi empregado em um micro-arranjo de tecido (TMA) contendo 320 carcinomas mamários. Onze diferentes sistemas de visualização foram utilizados: seis SPLB de segunda geração (Advance, Novolink, SuperPicTure, PicTure Max, Super Sensitive Non-Biotin HRP, e Mouse/Rabbit Polydetector HRP/DAB), um SPLB de primeira geração (EnVision+), e quatro SABS (LSAB+, EasyPath, Super Sensitive, e Mouse/Rabbit Immunodetector HRP/DAB). As lâminas foram digitalizadas usando o Mirax Scanner e as imagens obtidas foram analisadas de forma automatizada e de forma visual adotando o sistema de escore de Allred para marcação nuclear. Marcação citoplasmática foi avaliada de forma semi-quantitativa. **Resultados:** Os SPLB Advance e Novolink mostraram os escores mais altos na análise visual e ainda detectaram dois casos positivos que foram considerados negativos utilizando outros sistemas de visualização. Estes sistemas, juntamente com o SAB LSAB+, também mostraram intensidade de marcação mais forte pela análise automatizada. SPLB não mostraram marcação citoplasmática, ao contrário dos SABS. **Conclusão:** os SPLB de segunda geração, especialmente o Advance e o Novolink, garantem sinal imuno-histoquímico mais intenso e marcação nuclear mais bem localizada, sem

coloração citoplasmática, quando comparados aos SABS. Outros estudos correlacionando estes achados com a condição de resposta terapêutica das pacientes devem ser realizados já que houve discordância quanto à positividade em alguns casos. SPLB representam uma ferramenta de alta qualidade para pesquisa e avaliação clínica do receptor de estrógeno em câncer de mama.

SUMMARY

Aims: Biotin-free polymeric visualization systems (BFPS) were compared to streptavidin-biotin systems (SABS) in the evaluation of immunoreactivity for estrogen receptor (ER) in breast carcinomas. **Methods:** The anti-estrogen antibody clone SP1 was employed on a tissue microarray containing 320 breast carcinomas. Eleven different detection systems were used: six BFPS of second generation (Advance, Novolink, SuperPicTure, PicTure Max, Super Sensitive Non-Biotin HRP, and Mouse/Rabbit Polydetector HRP/DAB), one BFPS of first generation (EnVision+), and four SABS (LSAB+, EasyPath, Super Sensitive, and Mouse/Rabbit Immunodetector HRP/DAB). The slides were digitalized using the Mirax scanner and the resulting images were analyzed both by an automated method and by visual analysis using the Allred´s score system considering positive nuclear staining. Cytoplasm staining was also separeted evaluated. **Results:** The BFPS Advance and Novolink showed the highest scores by visual analysis, and additionally detected two positive cases which were considered negative using the other detection systems. Likewise, these systems, together with the SAB LSAB+, showed higher staining intensity by the automated method. BFPS revealed no cytoplasm staining, in opposition to the SABS. **Conclusions:** The second generation BFPS, especially Advance and Novolink, provide stronger and sharper nuclear immunohistochemical signal as compared to most SABS, without nonspecific cytoplasm staining. As in few instances these detection systems show discordant results in relation to SABS, further studies

correlating these findings to therapeutic response are necessary. BFPS may represent a high quality tool both for research and clinical evalutation of estrogen receptor in breast cancer.

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LISTA DE ABREVIATURAS

IHC	*Immunohistochemistry*
BFPS	*Biotin-free polymeric systems*
SABS	*(Strept)avidin-biotin systems*
HER2	*Human epidermal growth factor receptor type 2*
EGFR	*Epidermal growth factor receptor*
ER	*Estrogen receptor*
PR	Progesterone receptor
DNA	Desoxiribonucleic acid
TMA	*Tissue microarray*
UK-NEQAS	*United Kingdom National External Quality Assessment Service*
cDNA	DNA complementar
FISH	*Fluorescent in situ hybridization*
CISH	*Chromogenic in situ hibridization*
SISH	*Silver in situ hibridization*
IHQ	*Imuno-histoquímica*
HRP	*Horseradish peroxidase*
DAB	*Diaminobenzidine*
FAPEMIG	Fundação de Amparo à Pesquisa do Estado de Minas Gerais
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CAPES	Coordenação de Aperfeicoamento de Pessoal de Nível Superior
UFMG	Universidade Federal de Minas Gerais
UCL	University College London
UCLAD	University College London Advanced Diagnostics
UNICAMP	Universidade Estadual de Campinas
TRIS-HCl	Ácido clorídrico tris
CAP	Colégio Americano de Patologistas

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Introdução

1. O câncer de mama e a sua importância

O câncer de mama é considerado o segundo tipo de câncer mais comum no mundo, sendo o mais freqüente entre as mulheres. Ao contrário do câncer de colo de útero, esta doença encontra-se relacionada ao processo de industrialização, com risco de adoecimento associado a elevado status sócio-econômico, além de outros fatores de risco clássicos descritos, tais como baixa paridade, idade precoce da menarca e tardia da menopausa, obesidade, altura e consumo de álcool. O câncer de mama é um importante problema de saúde pública na América Latina, tendo sido observado aumento consistente nas taxas de mortalidade nos últimos quarenta anos. Estudo conduzido no estado de São Paulo identificou o câncer de mama como a principal causa de mortalidade por neoplasias em mulheres na faixa etária de 30 a 49 anos. De acordo com estudo caso-controle conduzido em hospital universitário de Belo Horizonte, o perfil de risco para o câncer de mama relativo a fatores sócio-econômicos e reprodutivos apresentou-se similar em mulheres com diagnóstico efetuado na pré e na pós menopausa. A partir de estudo ecológico realizado com dados de exposição a pesticidas em onze estados do Brasil, foram identificadas correlações importantes entre esta exposição e distúrbios no sistema reprodutivo humano, incluindo a mortalidade por câncer de mama, principalmente em mulheres com idade entre 50 a 69 anos no período de 1995 a 1997. No entanto, estudo caso-controle realizado na Região Metropolitana do Rio de Janeiro, incluindo 177 casos e 350 controles, não verificou associação entre nível sérico de

organoclorados e aumento de risco para o câncer de mama. Com relação ao rastreamento das neoplasias malignas de mama, observou-se que mulheres idosas e mulheres com baixo grau de escolaridade apresentaram menores oportunidades de diagnóstico precoce. As mais elevadas taxas de incidência anuais de câncer de mama no Brasil, ajustadas por idade e por 100 mil mulheres, foram encontradas em São Paulo (94,0), Distrito Federal (86,1) e Porto Alegre (66,5) (GUERRA et al. 2005).

2. Fatores prognósticos e preditivos em câncer de mama

Fatores prognósticos são achados clínicos, patológicos e biológicos do paciente e do tumor que são úteis para predizer o comportamento clinico do paciente, como possibilidade de recorrência da doença ou sobrevida do paciente se este não for tratado (ALLRED et al. 1998). Dentre os principais fatores prognósticos, temos tamanho do tumor, tipo e grau histológico, numero de mitoses e metástase. Fatores preditivos são achados clínicos, patológicos e biológicos usados para estimar a resposta do paciente a um tipo especifico de terapia. Os principais marcadores imunohistoquímicos com importância preditiva e prognostica são receptores hormonais, Ki67 e HER2. Estes fatores são avaliados por patologistas, que fornecem os valores preditivos e prognósticos juntamente com as características morfológicas do câncer de mama enquanto oncologistas utilizam estas interpretações, em associação com outros dados clínicos, para a tomada de decisão terapêutica (GOBBI et al. 2008).

Em 1999, reunião de consenso no Colégio Americano de Patologistas (CAP) estratificou os fatores prognósticos do câncer de mama em três categorias especificadas no Quadro 1 (FITZGIBBONS et al. 2000). A categoria I inclui os fatores já validados como importantes no prognóstico e úteis na conduta clínica dos pacientes. Na categoria II foram englobados os fatores prognósticos que têm sido intensamente estudados do ponto de vista da biologia e da clínica, mas que ainda precisam ser validados em estudos estatísticos consistentes. A categoria III inclui todos os outros fatores ainda não suficientemente estudados para demonstrar seu valor prognóstico. O tipo e grau histológicos dos tumores têm seu valor bem estabelecido e foram incluídos na categoria I do CAP. Estudos de longos seguimentos mostram que alguns carcinomas de tipo especial cursam com melhor prognóstico (por exemplo: tubular, mucinoso e cribriforme) enquanto outros indicam prognóstico desfavorável (por exemplo: ductal SOE e apócrino) (PAGE et al. 1998).

QUADRO 1 – Categorias de fatores prognósticos em câncer de mama segundo a conferência de consenso de 1999 do Colégio Americano de Patologistas

Categoria	Fatores prognósticos
	Estadiamento (TNM)
	Grau histológico
I	Tipo histológico
	Número de mitoses
	Receptores hormonais
	HER-2/neu (c-erbB-2)
	p53
II	Invasão vascular linfática ou sangüínea
	Marcadores de proliferação celular: MIB-1
	Análise de DNA: fração de fase S
	Análise de ploidia de DNA
	Angiogênese tumoral
	EGFR
III	TGF-α
	bcl-2
	PS2
	Catepsina D

FITZGIBBONS, *et al.*, 2000.

O grau histológico pode também ter valor preditivo no que diz respeito à resposta à quimioterapia. Tumores de alto grau em geral apresentam melhor resposta a determinados quimioterápicos quando comparados com tumores de baixo grau histológico (POPE et al. 2006).

Todo fator preditivo de resposta terapêutica deve ser validado técnica e clinicamente bem como influenciar na tomada de decisão clinica. Uma validação técnica significa que o método usado para a avaliação do fator preditivo seja sensível, específico, reprodutível, e possa ser interpretado de maneira universal em diferentes laboratórios. A validação clinica consiste no fato de que o teste identifique diferentes grupos de pacientes com diferentes riscos de recorrência da doença, tempo de sobrevida e resposta terapêutica.

Embora fatores preditivos de resposta terapêutica tenham maior valor clínico, os únicos fatores preditivos amplamente validados na literatura para a rotina clínica são receptor de estrógeno, progesterona e HER2.

2.1- Receptor de estrógeno

Receptor de estrógeno é provavelmente o fator preditivo individual mais importante avaliado em câncer de mama. Até dois terços das mulheres acima de 50 anos apresentarão câncer de mama positivos para receptor de estrógeno, além disso, aproximadamente 80% dos tumores de mama em mulheres acima de 50 anos são positivos para receptor de estrógeno. Receptor de estrógeno é importante no processo de carcinogênese e sua inibição, seja diretamente utilizando agonistas fracos de estrógeno (Moduladores Seletivos do Receptor de Estrógeno) ou indiretamente através do bloqueio da conversão de androgênios em estrógeno (inibidores de aromatase), é considerada a base da terapia adjuvante do câncer de mama. Aos tumores que são positivos para receptores de estrógeno ou progesterona são atribuídas características de baixo risco de

mortalidade para o paciente comparados aos negativos para os mesmos receptores (PAYNE, 2008).

A exposição ao estrógeno é conhecidamente um fator de risco para o câncer de mama (COLDITZ, 1998). O hormônio exerce seus efeitos biológicos através do seu receptor, que são proteínas nucleares classificadas em dois subtipos: receptor de estrógeno alfa e beta (NILSSON et al. 2001). Ambos são fatores de transcrição que mediam a ação do estrógeno. Os receptores alfa e beta se ligam ao estradiol na mesma extensão, porém se diferenciam na afinidade com a qual se ligam e também na resposta produzida subseqüentemente (WATANABE et al. 1997; BARKHEM et al. 1998). O receptor de estrógeno alfa foi identificado primeiro e, então, renomeado receptor de estrógeno alfa a partir da descrição do subtipo beta. O receptor de estrógeno alfa é expresso em ate 80% de todos os cânceres de mama. No entanto, seu valor preditivo não é ideal até o momento, já que aproximadamente um terço dos cânceres de mama metastáticos positivos para este receptor não apresentam resposta a terapia hormonal (OSBORNE, 1998).

2.2- Receptor de progesterona

A expressão do receptor de progesterona parece indicar a funcionalidade da ação do receptor de estrógeno (HORWITZ et al. 1978). Portanto, a avaliação do receptor de progesterona deveria, teoricamente, ajudar a prever a resposta a hormonioterapia de maneira mais apurada. Há evidencias de que tumores positivos para receptor de progesterona apresentam maior probabilidade de

responder ao tamoxifen tanto em pacientes com neoplasia metastática (RAVDIN et al. 1992) quanto no tratamento adjuvante (BARDOU et al. 2003). O receptor de progesterona pode ser detectado em casos negativos para receptor de estrógeno em até 5%. Este fato poderia caracterizar um exame falso negativo para receptor de estrógeno, presença muito baixa deste receptor nas células neoplásicas, ou até mesmo variantes de receptor de estrógeno não reconhecidos pelo anticorpo, mas efetivo na estimulação da expressão de receptor de progesterona (FUQUA et al. 1991). Ainda há controvérsia quanto ao valor preditivo da positividade do receptor de progesterona na vigência de negatividade do receptor de estrógeno. Alguns estudos sugerem que tumores receptor de progesterona positivo, mesmo negativos para receptor de estrógeno, determinam grupo de pacientes mais responsivos à hormonioterapia, porém estes achados não são universais (BARDOU et al. 2003).

2.3- Receptor do Fator de Crescimento Epidérmico Humano Tipo 2 (HER2)

O HER2, também conhecido como c-erb-B2, ERBB2 ou HER2/*neu*, é um membro da família dos Receptores do Fator de Crescimento Epidérmico (EGFR) de tirosinoquinases e está localizado no cromossomo 17q21. Este receptor é importante para a diferenciação celular, adesão e mobilidade (HANNA et al. 1999). A amplificação é o mecanismo predominante da superexpressão gênica e, conseqüentemente, altos níveis de seu produto protéico de 185 KDa são evidenciados em aproximadamente 18 – 20% dos cânceres de mama (SLAMON et al. 1987). A positividade do HER2 está associada com tumores de alto grau,

envolvimento linfonodal (BURSTEIN 2005), alto índice de recorrência tumoral e mortalidade, portanto, está relacionado a mau prognostico (YAMAUCHI et al. 2001).

A avaliação do HER2 em câncer de mama também representa fator preditivo de resposta terapêutica, mais especificamente ao trastuzumab (Herceptin®; Genetech, Sao Francisco, Califórnia, EUA). Esta droga consiste em um anticorpo monoclonal humanizado, que tem mostrado melhores respostas ao tratamento do câncer e maior tempo de sobrevida da paciente livre de doença, tanto ao ser administrado sozinho, ou em combinação com quimioterápicos em pacientes com neoplasia metastática (SLAMON 2001). Mais recentemente, cinco estudos clínicos internacionais prospectivos e randomizados mostraram que o trastuzumab, ao ser administrado de forma adjuvante, reduz significantemente o risco de recorrência e mortalidade em pacientes com estágio precoce da doença (ROMOND et al. 2008; JOENSUU et al. 2006; SMITH et al. 2007). Além disso, esta droga foi recomendada pelo *National Institute for Clinical Excellence* (NICE) no Reino Unido para o tratamento de pacientes em estágio precoce e metastático do câncer de mama. Recentemente, a utilização de inibidor de tirosinoquinases, o lapatinib (Tykerb; GlaxoSmithKline, Philadelphia, Pensilvânia, EUA) tem mostrado resultados promissores em cânceres de mama HER2 positivos, em combinação com capecitabine (GEYER et al. 2006).

HER2 pode ajudar a prever a resposta terapêutica a diferentes agentes quimioterápicos Por exemplo, alguns estudos sugerem que tumores positivos para HER2 podem ser mais sensíveis a antraciclinas (KONECNY et al. 2004;

PRITCHARD et al. 2006). Sugere-se ainda que a positividade para HER2 pode predizer resistência à terapia endócrina, porém os estudos que mostram este fato são retrospectivos e não empregaram populações comparáveis, nem metodologia semelhante para detecção imuno-histoquímica dos marcadores. Até o momento, a positividade do HER2 não é utilizada para decidir ou não sobre o emprego da terapia endócrina (PROWELL & ARMSTRONG 2006).

Testes apurados e precoces para HER2 de todos os pacientes portadores de câncer de mama são essenciais para o aperfeiçoamento do tratamento. Testes errôneos ou inconclusivos para HER2 podem resultar em inapropriada conduta clínica e irão apenas dificultar a tomada de decisão sobre a utilização de novas drogas para o tratamento do câncer de mama (DENT & CLEMONS 2005).

3- Metodologia de avaliação de receptores hormonais

Imuno-histoquímica é o método padronizado para a determinação do *status* dos receptores hormonais (ROCHA et al. 2009; ROCHA et al. 2008; GOBBI et al. 2008) e pode ser aplicada a especimes de *core biopsy* e excisões cirúrgicas. Há dados do *National Health Service* (NHS) do Reino Unido que asseguram que a imuno-histoquímica pode ser altamente reprodutível entre diferentes laboratórios. Baixa qualidade de fixação do tecido pode afetar os resultados da técnica, e controles positivos e negativos devem ser usados para cada bateria de testes (PAYNE et al. 2008).

É necessário que o nível de imunorreatividade do tumor para receptor de estrógeno e progesterona seja reportado, já que tumores não reativos para tais hormônios não responderão a terapia endócrina. Aqueles pacientes cujos tumores mostram baixos valores de avaliação para receptor de estrógeno, porém altos valores para receptor de progesterona, também se beneficiarão com a terapia hormonal. Apesar de vários sistemas de escore serem conhecidos atualmente (LEAKE et al. 2000; REINER et al. 1990), com o objetivo de obter melhor padronização interlaboratório é recomendado que o método de escore de Allred seja utilizado (GOLDHIRSCH et al. 2005). Este método combina a intensidade de marcação imuno-histoquímica e a proporção de células marcadas, resultando em um escore máximo de oito (Tabela 1). Estudo correlacionando a resposta terapêutica com o nível de expressão de receptor de estrógeno avaliado por este sistema de escore mostrou que mesmo pacientes cujos tumores que receberam um total de três podem se beneficiar da terapia endócrina adjuvante (HARVEY et al. 1999).

Tabela 1: Sistema de escore proposto por ALLRED (1999) para avaliação de receptor de estrógeno em câncer de mama obtido pela combinação de intensidade de marcação e proporção de células marcadas

Escore de intensidade	Escore de proporção de células marcadas (%)				
	0	**> 0 a 10**	**> 10 a 33**	**> 33 a 66**	**> 66 a 100**
Fraco	0	2	4	5	6
Moderado	0	3	5	6	7
Forte	0	3	6	7	8

* O escore total é obtido através da soma do escore de proporção de células marcadas com o escore de intensidade de marcação. Escores totais entre 0 e 2 são considerados negativos e escores totais acima de 2 são considerados positivos.

4- Controle de qualidade em imuno-histoquímica

É recomendável que qualquer teste utilizado para propósitos clínicos deva ser oferecido por laboratórios que sejam assegurados com certidão de alta qualidade tanto por fiscalização interna, controle interno de qualidade, quanto pela participação de um programa de controle externo da qualidade imuno-histoquímica (ROMAIN et al. 1995; KOENDERS & THORPE 1983).

4.1- Controle interno de qualidade em imuno-histoquímica

Procedimentos internos de controle de qualidade devem ser adotados pelos laboratórios que realizam imuno-histoquímica considerando-se vários critérios, que incluem a padronização do método. Estes procedimentos, além de fazerem parte do controle interno de qualidade do laboratório, podem ser avaliados por serviços de avaliação externos como o *Clinical Pathology Accreditation* (CPA), Reino Unido. Também é esperado que os laboratórios de anatomia patológica fiscalizem parte de sua própria produção diária, e este passo já é adotado em grande parte dos laboratórios. Por exemplo, o laboratório pode fiscalizar uma proporção de biópsias selecionadas de maneira aleatória (MAXWELL & McCLUGGAGE 2000). Durante esta fiscalização, serão avaliados quesitos como veracidade dos dados do paciente, *turnaround time*, qualidade da marcação imuno-histoquímica e representatividade da amostra tumoral submetida ao teste (LIND et al. 1995; PRESCOTT et al. 1995; RAMSEY 1999). É esperado que os laboratórios sigam protocolos de padronização para garantir que a reação imuno-histoquímica seja de alta qualidade. Desta maneira, anticorpos que apresentarem marcação aquém do esperado possam ser identificados e providências serem tomadas, como a modificação de protocolos ou até a exclusão daquele anticorpo da rotina do laboratório (MAXWELL & McCLUGGAGE 2000). Até o momento, pouco foco tem sido dado à qualidade da reação imuno-histoquímica em si e, com exceção da avaliação do desempenho da rotina e checagem de positividade e negatividade dos controles.

Há poucas recomendações na literatura a respeito de controle interno de qualidade em imuno-histoquímica (ROCHA 2009).

4.2- Controle externo de qualidade em imuno-histoquímica

No Reino Unido, o *United Kingdom National External Quality Assessment Service for Immunohistochemistry* (UK-NEQAS-ICC) é responsável pela coordenação do controle externo de qualidade em imuno-histoquímica em geral e, mais recentemente, FISH para HER2. Este programa foi fundado em 1985 e, em 1988, o programa foi reconhecido pelo Departamento de Saúde do Reino Unido. A partir disto, o programa passou a ser denominado United Kingdom National External Quality Assessment Service for Immunocytochemistry (UK NEQAS-ICC) e mais recentemente, quando a hibridação *in situ* foi incluída no programa, passou a ser denominado: UK NEQAS-ICC & ISH. O número de laboratórios inscritos no programa aumentou muito nos últimos três anos. Ao longo destes 20 anos tem ocorrido uma melhoria progressiva na qualidade das colorações imuno-histoquímicas. Atualmente, mais de 70% dos laboratórios produzem resultados aceitáveis para avaliação de receptor de estrógeno em lâminas fornecidas pelo NEQAS contendo células de cultura. O NEQAS requer que cada laboratório core secções de três tumores cujo *status* de receptor de estrógeno já havia sido avaliado. As lâminas coradas são retornadas para serem avaliadas por quatro dos avaliadores do NEQAS. Aos participantes também é solicitado que se envie um exemplo de uma secção de tumor corada e seu

diagnóstico dado pelos analisadores do laboratório. 97% dos laboratórios produzem colorações classificadas como aceitáveis em suas próprias secções, mostrando que cada um tem adaptado eficientemente seus protocolos para suas próprias condições.

Diversas razões técnicas, incluindo o método de reativação antigênica, a concentração do anticorpo primário e a sensibilidade do sistema de visualização podem causar variações importantes no resultado final da imuno-histoquímica. Portanto, todas estas variações devem ser controladas cuidadosamente e o uso de controles positivo e negativo é, então, imprescindível. O controle positivo deve conter múltiplas amostras previamente avaliadas contendo diferentes níveis de marcação para o anticorpo em teste. O uso de tecido com baixo escore prévio para receptor de estrógeno é particularmente importante para se checar a sensibilidade do método empregado e faz parte dos procedimentos de controle interno do laboratório (BARNES et al. 1998). No entanto, a participação em programas de controle externo são fundamentais tanto para o aprimoramento técnico laboratorial quanto para o treinamento interpretativo dos resultados obtidos na reação.

5- A importância das fases pré-analítica, analítica e pós-analítica

A imuno-histoquímica envolve as etapas pré-analítica, analítica e pós-analítica. A fase pré-analítica compreende a fase de preparação do tecido e tem como principal etapa a fixação. Problemas relacionados a esta etapa são os

principais responsáveis por baixa qualidade da reação imuno-histoquímica por levar a marcações inespecíficas, danos na morfologia do tecido ou mesmo ausência de marcação. Uma fixação ideal deve levar em consideração o tipo de fixador, o seu tamponamento em pH ideal e o tempo de fixação do tecido (ROCHA et al. 2008).

A fase analítica compreende as fases técnicas do ensaio imunoistoquímico em si. Os problemas relacionados à fase analítica ou de processamento da reação são os principais responsáveis por baixas avaliações nos programas externos de controle de qualidade (HAMMOND et al. 2003; RHODES et al. 2002; SEIDAL et al. 2001; WICK et al. 2002). Os principais fatores a serem levados em consideração nesta etapa seriam: a escolha do anticorpo primário e sua diluição, a escolha do método de reativação antigênica e seus reagentes e, por fim, a escolha do sistema de visualização imuno-histoquímica a ser utilizado.

A fase pós-analítica compreende a interpretação e quantificação dos resultados. Falhas na interpretação da reação levam a dificuldade de padronização da imuno-histoquímica e discordâncias entre laboratórios como observado em programas de controle de qualidade externo (MILLER 2008).

Todas as etapas são importantes e ainda há grande variação nas metodologias empregadas pelos diferentes laboratórios. Como resultado, a padronização do método é variável e, ao se tratar de testes preditivos, irá influenciar nas decisões terapêuticas de cada paciente.

6 - A tecnologia dos microarranjos de tecido (tissue microarrays, TMA)

O advento de estudos moleculares baseados no genoma de doenças levou à identificação de centenas de moléculas potencialmente associadas com o processo de doença. Surgiu então, a necessidade de analisar melhor estas moléculas em amostras de tecido para elucidar sua importância como biomarcadores ou alvos fisiológicos de prevenção baseados em drogas ou tratamentos específicos. O TMA tem sido considerado uma etapa necessária à consolidação do conhecimento adquirido através do mapeamento genético dos estudos realizados com a técnica de “cDNA Microarrays”. Esta técnica determina quais as dezenas ou centenas de genes estão superexpressos ou hipoexpressos em um tumor e que poderiam ser responsabilizados por alguma particularidade biológica, tais como o rápido crescimento, o potencial de recorrência local ou de metástase à distância, ou ainda, resistência à quimioterapia (SAUTER et al. 2002).

A técnica de *tissue microarray* (TMA) permite a análise retrospectiva em larga escala da expressão protéica dos tumores através da imuno-histoquímica, sendo possível conhecer a expressão de uma determinada proteína em até 1000 casos diferentes em apenas uma lâmina histológica. O TMA apresenta acurácia comprovada que justifica seu emprego com amplas vantagens de custo e tempo para os estudos retrospectivos de grandes centros ou estudos

cooperativos com grandes bancos de dados (BUBENDORF et al. 2001; MILANES – YEARSLEY et al. 2002; SAPINO et al. 2006).

Em 1986, foi desenvolvida a técnica do *sausage ou multi-tissue tumor block* (MTTB) (BATTIFORA et al. 1986), onde tecidos diferentes eram processados juntos em um mesmo bloco de parafina de modo aleatório e não ordenado. Esses blocos eram utilizados para teste de novos anticorpos e como controle de qualidade das reações imuno-histoquímicas. Outros autores aprimoraram esta técnica: cilindros de amostras eram retiradas de tecidos de blocos de parafina pré-existentes e reembebidos em um novo bloco de forma ordenada, com coordenadas pré-estabelecidas, tamanhos e formas regulares (KONONEN et al. 1998). Havia um maior número e melhor preservação das amostras e dos blocos, permitindo o estudo de um grande número de casos analisando-se apenas uma ou poucas lâminas. Essa técnica adicionou a vantagem de todas as amostras serem processadas em um mesmo momento e em condições idênticas. Ainda, preserva o material remanescente para outras pesquisas ou necessidades diagnósticas.

6.1- A construção dos TMA

Para a construção do TMA o pesquisador estuda as lâminas originais e seleciona as áreas de interesse que representem o tumor. A seguir as áreas selecionadas são identificadas nos blocos de parafina arquivados (bloco doador) e um cilindro de tecido (que pode variar de 0,6mm a 2,0mm de diâmetro e 3 a 4

mm de comprimento) é retirado desta área com uma agulha acoplada a um equipamento de precisão. Este cilindro é então introduzido em um novo bloco (bloco receptor), previamente preparado com orifícios cilíndricos vazios. Os cilindros dos vários casos são sucessivamente adicionados ao bloco receptor, manualmente ou com a ajuda de aparelhos específicos, e a posição de cada caso é identificada em uma planilha com referências de coluna e linha (eixos X e Y). Ao fim, obtém-se um bloco receptor que pode conter até 1000 amostras diferentes. Deste bloco são obtidos cortes histológicos seqüenciais numerados em lâminas tratadas com adesivo que permitem a realização de reações imuno-histoquímica, FISH, SISH ou CISH. É importante registrar o conjunto de fatores histológicos relevantes e, eventualmente, prognósticos dos tumores selecionados (idade do paciente, grau e diferenciação histológicos, grau nuclear, margens, invasão vascular, etc.) e reclassificar as doenças quando necessário. Este deve ser feito em arquivo ou planilha permanente para armazenar todas as informações referentes a cada um dos cilindros que irão compor o TMA, com suas coordenadas X e Y no bloco receptor (TROTER et al. 2002).

A leitura das lâminas dos TMA pode ser feita em microscopia óptica convencional pelo pesquisador, seguindo a planilha previamente preparada com as coordenadas X e Y, anotando os resultados encontrados em papel ou diretamente na base de dados do computador. Vários grupos desenvolveram métodos de arquivo digital de imagens com softwares de captura de imagens, interpretação e armazenamento automatizado dos dados, permitindo rápida correlação entre os dados armazenados previamente e os resultados obtidos,

revisão das imagens, quando necessário, estudos comparativos de reprodutibilidade, etc. (LIU et al. 2002; MANLEY et al. 2001).

6.2 – TMA e câncer de mama

Os estudos para validação do método TMA no carcinoma da mama demonstraram ser possível atingir 98% de concordância com as reações nos cortes histológicos usuais para marcadores imunoistoquímicos como o receptor de estrógeno e HER2, utilizando-se três amostras de 0,6mm de diâmetro de cada caso. Quando se utilizaram apenas duas amostras a concordância obtida foi de 94% (CAMP et al. 2000; De MARZO & FEDOR 2004). Apesar da amostragem de mais de uma área nos carcinomas de mama ser importante para que nos TMA esteja representada toda a heterogeneidade destes tumores, estudos recentes mostraram alta concordância entre uma amostra de 0,6mm ou 2,0mm e os cortes habituais do bloco doador, e que a heterogeneidade intratumoral não altera o status do HER2 utilizando as técnicas IHQ, CISH e FISH (HENRIKSEN et al. 2006; LORING et al. 2005; SELVARAJAN et al. 2006; ZHANG et al. 2003).

Atualmente o TMA tem sido amplamente utilizado para testes de validação diagnóstica interlaboratoriais, principalmente FISH, CISH E imuno-histoquímica para a detecção do status de receptores hormonais e HER2 em grande número de casos, com padronização de protocolos das reações (DIAZ et al. 2004; KAY et al. 2004; SAEZ et al. 2006) e para estudos comparativos entre

diferentes testes diagnósticos para amplificação gênica e superexpressão protéica do HER2, bem como para a avaliação de receptores hormonais em câncer de mama (ZHANG et al. 2003).

7 - A tecnologia de digitalização de lâminas histológicas

Apesar de muitos avanços no entendimento da evolução dos tumores, os aspectos mais básicos da Anatomia Patológica têm se modificado de forma relativamente modesta ao longo dos anos. O patologista supervisiona a macroscopia do material fixado, desidratação em solventes orgânicos, inclusão em parafina, microtomia e coloração. O tecido é então, usualmente, avaliado utilizando um microscópio de luz comum. Então, o patologista libera um diagnóstico e a lâmina e o bloco são encaminhados para o arquivo. Portanto, a distribuição de material para consultoria, pesquisa ou ensino se torna laboriosa e os patologistas restringem seu acesso apenas a material de casos disponíveis localmente.

O termo “telepatologia” foi usado pela primeira vez em 1986 e, desde então, tem-se notado aumento de interesse em tecnologias que tornem possível a idéia de avaliar espécimes a distância. Até os anos de 1990, havia duas formas de telepatologia: estática e dinâmica. Em telepatologia estática, o patolgista capturava um pequeno número de imagens que eram transmitidos via internet e analisadas em tempo real. A forma dinâmica podia ser realizada utilizando um microscópio controlado remotamente por um patologista a

distância. O patologista consultor seria capaz de controlar o microscópio e selecionar as imagens a serem vistas. Alguns microscópios ofereciam a função de troca de filtros de diferentes cores ou aplicar diferentes modos de iluminação. Alguns sistemas desta natureza ainda permitiam a análise simultânea por vários observadores desde que apenas um comandasse o microscópio.

A nova era da telepatologia trouxe um novo conceito tecnológico: “as lâminas digitais”. Lâminas digitais são espécimes microscópicos virtuais inteiros que podem ser vistos e analisados pelo monitor de um computador. As lâminas digitais podem ser acessadas pelo disco de memória do computador, CD-ROM, DVD, discos removíveis ou mesmo de uma rede de servidor utilizada para arquivamento de imagens e distribuição. Imagens digitais de campos adjacentes em lâminas de cito e histopatologia são colocadas juntas, em ordem, para formar uma montagem de imagem gigante, utilizando uma plataforma escaneadora (digitalizadora) controlada por computador. Embora esta tecnologia já exista há mais de uma década, este processo, também chamado de digitalização de lâmina inteira, se tornou disponível comercialmente para computadores comuns apenas em 1998, quando suficiente memória e velocidade de processamento de dados também se tornaram disponíveis. Já que o observador pode explorar o corte histológico inteiro, as lâminas digitais substituem, com vantagens, as lâminas reais para fins educacionais, consultoria a distância, e armários cheios de lâminas armazenadas. Recuperar uma lâmina do arquivo se torna apenas uma questão de acessar um banco de dados virtual. Além disso, são possíveis acessos simultâneos a uma lâmina digital por

diferentes observadores, localizados a distância (diferentes países, por exemplo). Novos programas de computador estão sendo desenvolvidos e podem desempenhar diversas funções como reconstrução de imagem em três dimensões a partir de imagens obtidas pelo escaneador naquele mesmo campo visual em múltiplos planos focais, segmentação de imagens, reconhecimento de padrões morfológicos e recuperação de imagens (lâminas digitais) que apresentem as mesmas estruturas identificadas em uma lâmina teste. Lâminas digitais podem ainda ser usadas como importante ferramenta em programas de controle externo de qualidade laboratorial. Soluções técnicas para um sistema de visualização de lâminas digitais através da internet já foram desenvolvidas e alguns exemplos estão disponíveis para avaliação pública.

O desenvolvimento de programas de computador para visualização e análise das lâminas virtuais, capacidade de armazenamento virtual e também dedicação, por parte dos fabricantes dos equipamentos, em promover treinamentos para os usuários parecem representar os maiores desafios desta nova tecnologia para os próximos anos (ROCHA et al. 2009).

8 – Aspectos éticos

Este estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais sob número de processo: 510 / 04

Objetivos

Objetivos

Os objetivos do nosso estudo, bem como material e métodos, resultados e discussão serão apresentados sob forma de trabalhos científicos aceitos em periódico nacional e internacionais como segue especificado abaixo:

1 – Comparar os polímeros livres de biotina e os sistemas avidina-biotina para a avaliação de receptor de estrógeno em câncer de mama através da quantificação automatizada de intensidade de coloração imuno-histoquímica utilizando microscopia virtual e softwares analisadores. (Applied Cancer Research – In press)

2 – Comparar os polímeros livres de biotina e os sistemas avidina-biotina para a avaliação de receptor de estrógeno em câncer de mama através do escore manual de Allred empregando microscopia virtual e comparar esta avaliação qualitativa com o valor numérico conseguido pela quantificação automatizada. (Journal of Clinical Pathology – In press)

3 – Fazer uma análise da atual importância e potencias da patologia digital em laboratórios de rotina e pesquisa em anatomia patológica, bem como para processos educacionais e programas de controle externo de qualidade em imuno-histoquímica (Pathology Research and Practice – In press; Immunocytochemistry – In press).

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The use of the immunohistochemical biotin-free visualization systems for estrogen receptor evaluation of breast cancer

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Abstract

Background: A novel generation of immunohistochemical visualization systems based on a biotin-free polymeric (BFP) technology has been released recently. We have compared the new BFP and the classical streptavidin-biotin systems (SAB) to evaluate estrogen receptor in breast carcinomas. Methods: Serial sections from a tissue microarray containing 320 invasive breast carcinomas were stained by immunohistochemistry for estrogen receptor using the rabbit monoclonal antibody SP1. Eleven different visualization systems were used, including 7 BFP (the 2nd generation Advance, DAKO™; Novolink, Leica™; SuperPicTure, Zymed™; PicTure Max, Zymed™; Super Sensitive Non-Biotin HRP, Biogenex™ and Mouse/Rabbit Polydetector HRP/DAB, CellMarque™; and the 1st generation EnVision+ DAKO™) and 4 SAB (LSAB+, DAKO™; EasyPath, Signet™; Super Sensitive, Biogenex™ and Mouse/Rabbit Immunodetector HRP/DAB, CellMarque™) following the instructions provided by the manufacturers. All slides were scanned using Mirax Scan, Zeiss™, and the intensity of immunohistochemistry staining was automated quantified using HistoQuant™ software. The cytoplasm staining was visually evaluated as absent (0), weak (1), moderate (2), or strong (3). Results: The BFP Advance and Novolink, and the SAB LSAB+ showed the highest staining intensity among all the systems ($P<0.01$). However, LSAB+ showed the highest cytoplasm staining among them ($p<0.01$). The other 2nd generation BFP showed similar staining intensities among them and also similar to the SAB. The 1st generation

EnVision+ showed the weakest staining intensity. The seven BFP showed sharper signal without cytoplasm staining compared to all SAB systems ($p<0.05$). BFP PicTureMAX showed the least cytoplasm staining. Conclusion: The BFP provide sharper and better localized immunohistochemical signal without cytoplasm staining compared to the SAB. The BFP Advance and Novolink showed the strongest staining intensity and, followed by all the other 2nd generation BFP, represent a powerful tool for immunohistochemistry standardization of estrogen receptor evaluation of breast carcinomas.

Key words: immunohistochemistry, automated image analysis, visualization systems

Introduction

The success of immunohistochemistry is largely a result of a development of reliable markers and of highly sensitive visualization procedures. The demonstration of antigens in tissues and cells by immunostaining is a two step process involving first, the binding of an antibody to the antigen of interest, and second, the detection and visualization of bound antibody by one of a variety of enzyme chromogenic systems. The choice of visualization systems will impact the sensitivity, utility, and ease-of-use of the method, including also reduction in incubation times [1].

Developments in immunohistochemistry techniques, especially antigen retrieval methods, and sensitive visualization systems, have allowed the detection of very small quantities of protein. The staining intensity depends on the staining procedures used in addition to the protein content itself [2]. The mostly used streptavidin-biotin visualization systems are based on the sequential application of biotinylated link antibody and streptavidin labeled with one or two molecules of alkaline phosphatase or peroxidase. These conventional biotin-rich procedures advertise the advantage that streptavidin presents high affinity for biotin [DakoCytomation Product Catalog 2005/2006].

Recently, a novel generation of polymeric biotin-free visualization systems has been released. The polymer-based, biotin-free detection reagent is based on polymeric technology, which uses compact enzyme-antibody conjugates. They are based in a polymeric HRP, which is a unique enzyme-conjugated polymer

backbone that also carries secondary antibody molecules [3]. The polymeric visualization systems are claimed to achieve signal amplification and thereby an enhanced sensitivity by increasing the number of enzyme molecules which are conjugated to the secondary antibody. According to the suppliers, endogenous biotin will not affect polymeric staining results due to their biotin-free characteristic.

In this study, we aimed to compare the performance of the biotin-free polymeric visualization system kits to the streptavidin-biotin-system kits for evaluating estrogen receptor using an automated image analysis system.

Material and Methods

Case Selection

Three hundred and twenty cases of invasive breast carcinomas, diagnosed between 1990 and 2005, were randomly selected from the files of the Breast Pathology Laboratory of Federal University of Minas Gerais, Medicine School of Campinas University and Cancer Hospital A.C. Camargo of São Paulo, Brazil. All original slides were reviewed to confirm the diagnosis and to select representative areas of tumors. One cylinder (1mm diameter) of each tumor with representative area of neoplasia was selected from paraffin blocks to build a tissue microarray (TMA). Two cylinders of tumors from previously tested and whose results were positive and negative were also included as internal controls for the TMA. Sequential 4µm sections were obtained and stained for hematoxylin

and eosin (first and last sections) to confirm diagnosis, and the interval sections were used for the immunohistochemical study. Slides containing full sections of previously tested positive breast tumor were included in all batches as external control.

Immunohistochemical Procedures

The sections were mounted on glass slides coated with silane (3-aminopropyltriethoxysilane) and dried for 30 minutes at 37ºC. The sections were deparaffinized in xylene and rehydrated via a series of graded alcohols. Endogenous peroxidase activity was blocked by following the procedures and the reagents supplied by each visualization system manufacturer. All sections were initially submitted to heat-induced epitope retrieval in steamer for 25 minutes under the same environment conditions. Each manufacturer supplied its antigen retrieval reagents, which were all citrate pH = 6.0. The rabbit monoclonal antibody SP1, Roche^{TM}, against estrogen receptor, was used as primary antibody for all the visualization systems. Preliminary testing was performed in our laboratory to identify the best concentration for the primary antibody and to choose the negative and positive controls using the dilution data supplied by the manufacturer as the starting point. The best primary antibody dilution achieved was 1:300 and this dilution was used for all the visualization systems for a 30 minute incubation. After washing the primary antibody with phosphate buffered saline (PBS), the slides were incubated with the reagents supplied by the manufacturers of each visualization system following all the procedures and

incubation times suggested in the specification sheets. Eleven different visualization systems were used, including seven biotin-free polymer systems (the 2nd generation: Advance, DAKO™; Novolink, Leica™; SuperPicTure, Zymed™; PicTure Max, Zymed™; Super Sensitive Non-Biotin HRP, Biogenex™ and Mouse/Rabbit Polydetector HRP/DAB, CellMarque™; and the 1st generation: EnVision+ DAKO™) and four streptavidin-biotin systems (LSAB+, DAKO™; EasyPath, Signet™; Super Sensitive, Biogenex™ and Mouse/Rabbit Immunodetector HRP/DAB, CellMarque™) as shown in Table 1. Freshly prepared DAB solution was applied following the procedures and incubation times suggested. DAB was removed by rinsing with distilled water. The slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted using Entelan™.

Table 1: Specification, type, and supplier of each visualization system

Visualization System	Type	Supplier
EnVision+	Biotin-free polymer 1st generation	Dako, Carpinteria, CA, USA
Advance	Biotin-free polymer 2nd generation	Dako, Carpinteria, CA, USA
NovoLink	Biotin-free polymer 2nd generation	Leica, New Castle, UK
SuperPicTure	Biotin-free polymer 2nd generation	Zymed, San Francisco, CA, USA
PicTure Max	Biotin-free polymer 2nd generation	Zymed, San Francisco, CA, USA
Super Sensitive non-biotin HRP	Biotin-free polymer 2nd generation	Biogenex, San Ramon, CA, USA
Mouse/Rabbit Polydetector HRP/DAB	Biotin-free polymer 2nd generation	CellMarque, Rocklin, CA, USA
LSAB +	Streptavidin-biotin based system	Dako, Carpinteria, CA, USA
EasyPath	Streptavidin-biotin based system	Signet, Dedham, MA, USA
Super Sensitive	Streptavidin-biotin based system	Biogenex, San Ramon, CA, USA
Mouse/Rabbit Immunodetector HRP/DAB	Streptavidin-biotin based system	CellMarque, Rocklin, CA, USA

Immunostaining Analysis

All slides submitted to immunohistochemistry were labeled, and the examiner was blinded to the visualization system used. The automated analysis was made using digital microscopy to quantify the intensity of immunohistochemical staining. All the slides were digitalized using Mirax Scan

(developed and produced by 3DHISTECH Ltd., Budapest, Hungary; distributed worldwide by Carl Zeiss, Jena, Germany) and the images provided by the software were exhibited in an LCD monitor under contrast, focus, saturation, and white balance standardization.

To evaluate the intensity of staining, the image analysis system HistoQuant™, 3DHISTECH™, Budapest, Hungary, was used (Figure 1). The software identified the immunohistochemical staining to be quantified by minimizing background-staining artifacts using image filters. Since the software recognizes the positive nuclei staining of all different intensities, the quantification was processed in each TMA spot automatically by the software and all the numerical data was exported to a Microsoft Excel file.

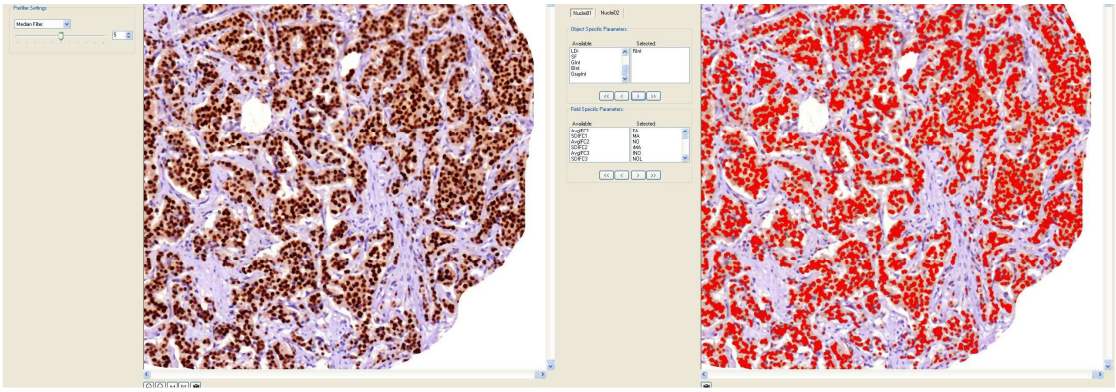

Figure 1: The same TMA spot in 200x magnification. A) Original immunohistochemical staining. B) Selected positive nuclei to have their intensity of immunohistochemical staining numerically quantified.

The cytoplasm staining was semi-quantitatively evaluated as absent (0), weak (1), moderate (2), and strong (3) by creating a TMA project, which exhibits each TMA spot at a time on the computer screen (Figure 2).

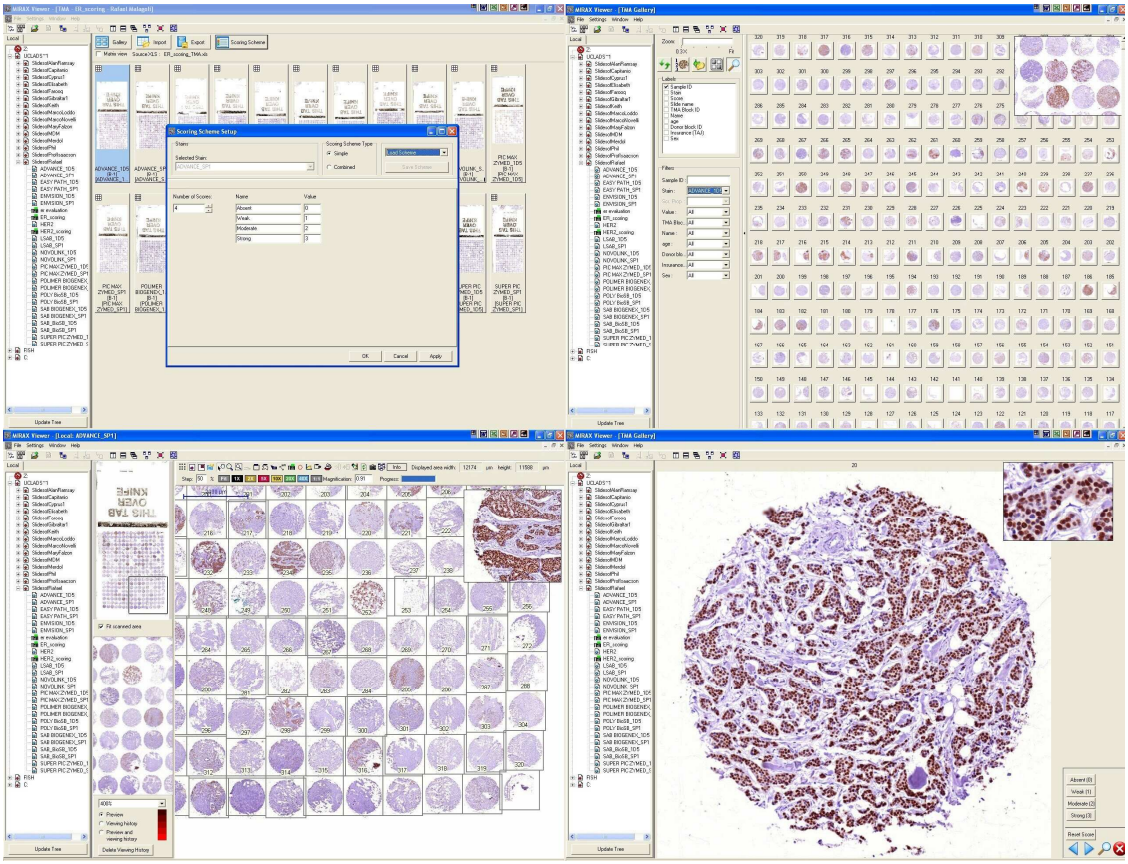

Figure 2: TMA project built in Mirax Viewer software. A) All slides in the background and a window containing the scoring scheme proposed for the background staining evaluation. B) 320 ordered and numerated TMA spots from one single slide shown individually in the screen. C) The same TMA spots shown in higher magnifications. D) One single TMA spot ready to be analyzed. The scoring scheme is shown in the picture footnote and a 400x magnification of this spot is shown in the window on the top.

Statistical Analysis

The Software WINKS – Statistical Data Analysis, Version 6.0, was used for the statistical analysis. Wilcoxon test was used to compare the different groups of paired variables. Chi-Square test was used to evaluate the difference in frequencies of score among the groups of categorical variables of the cytoplasm staining evaluation.

Ethics

The procedures described in this section are in compliance with the Helsinki Declaration and are also in accord with the ethical standards established by the institutions where this study took place.

Results

The biotin-free polymeric visualization systems Advance and Novolink showed the strongest staining intensity together with the SAB LSAB+, which showed stronger staining intensity among all streptavidin-biotin systems and also among most of the polymeric systems. The polymeric systems PicTure Max and Super Sensitive Non-Biotin HRP showed up intermediate staining intensity similarly to the streptavidin-biotin system Super Sensitive. The polymeric systems SuperPicTure and Mouse/Rabbit Polydetector HRP/DAB showed low intermediate staining intensity similarly to the streptavidin-biotin systems Mouse/Rabbit Immunodetector HRP/DAB and EasyPath. The polymer EnVision+ showed the weakest staining intensity among all the visualization systems.

All visualization systems and p values of statistical analysis are shown in Table 2 according to their intensity of immunohistochemical staining.

The polymeric systems showed a sharper nuclear signal with no cytoplasm staining when compared to streptavidin-biotin systems ($p<0.01$). The

biotin-free polymer PicTure MAX, Zymed™, showed the least cytoplasm staining and the streptavidin-biotin LSAB+ showed the most ($p<0.01$).

Table 2: Eleven different visualization systems grouped according their staining intensity level expressed by the p value

Staining intensity level	Biotin-free polymer system (p value*)	Streptavidin-biotin polymer (p value*)
Stronger	Advance (0.0034) NovoLink (0.0061)	LSAB+ (0.03)
Up intermediate	Super Sensitive non-biotin HRP (0.01) PicTure Max (0.01)	Super Sensitive (0.02)
Low intermediate	SuperPicTure (0.01) Mouse/Rabbit Polydetector (0.01)	Mouse/Rabbit imunodetector EasyPath
Weaker	EnVision+	---------

* p value of the statistical analysis between the staining intensity of each visualization system and the group of systems from the level below. There was no significant difference between the staining intensity of the polymeric systems and the streptavidin-biotin systems of the same level, neither between two systems of the same level group.

Discussion

The present study showed that highly sensitive visualization systems enhanced immunohistochemistry staining intensity. We have compared the kits of biotin-free polymeric visualization systems to the conventional streptavidin-

biotin-system kits to evaluate estrogen receptor in breast carcinomas. Due to the great number of different reagents and protocols from all the visualization system kits, every immunohistochemical reaction was carried out manually in the same environmental conditions. Since we wanted to compare the efficacy of the combination between the particular retrieval method and the visualization system provided by each kit, all the procedure steps, times and reagents supplied by each manufacturer were followed accordingly.

Interpretation of immunohistochemistry is usually done manually and is, therefore, dependent on the experience and ability of the interpreter. Computerized image analysis systems have been used since the late 1980s and were shown to provide a more accurate means of quantification of ER [14, 15]. Quantification of immunohistochemistry for ER using different types of image analysis has also shown strong agreement with manual scoring from experienced observers [16]. According to our automated quantification, the biotin-free Advance and Novolink showed the strongest staining intensity among the polymers. Advance, a ready-to-use, peroxidase-based visualization system is compatible with suitably diluted rabbit and mouse primary antibodies. According to its manufacturer, it is a super-sensitive, non-biotin based, immunohistochemical visualization system that is useful for the detection of antigens in low concentrations, for short incubation time or for higher dilution of primary antibodies. It consists of two main reagents: an HRP Link, which contains anti-mouse and anti-rabbit secondary antibodies in a Tris-HCl buffer and stabilizing protein and an anti-microbial agent, and an HRP Enzyme, which

contains antibodies polymerized with horseradish peroxidase in a Tris-HCl buffer and stabilizing protein and anti-microbial agent [DakoCytomation Product Catalog 2005/2006]. None of the biotin-free polymer systems employed in this study supply a link antibody between the primary and the polymer, except by Advance™ and PicTure Max polymer, Zymed™. This might be one reason for the increased staining intensity observed. Advance™ kit also supplies an enhancer reagent, which acts in DAB, and may provide improvements in sharpness and staining intensity. According to Leica datasheet, the polymer Novolink contains increased number of peroxidase molecules bound to an activated dextran backbone with lack of major gaps among them to improve chromogen reaction, and that might contribute for increasing staining intensity in this visualization system reactions. Novolink kit also supplies the Post Primary Block, which is used to enhance penetration of the subsequent polymer reagent and might be one reason for its high performance. On the other hand, the 1st generation polymer EnVision+, which does not supply a link antibody neither an enhancer, and yet shows larger gaps among its peroxidase molecules bound to the dextran backbone, showed the weakest staining intensity among the polymers tested in our study.

Our results show that some polymers present equivalent staining intensity to streptavidin-biotin systems according to the automated image analysis (Table 2). It is relevant to mention that the polymeric systems present macromolecules containing secondary antibodies and nearly 100 enzyme molecules polymerized in a dextram backbone, while the streptavidin biotin systems present up to four

enzyme molecules bound to one molecule of streptavidin. This great difference in molecule size might result in different membrane penetration capacities between these two groups of visualization systems. Although the presence of biotin is the cause of important background and cytoplasm staining in some tissues, the high affinity between streptavidin and biotin may be the most important reason for the strong staining intensity observed in some streptavidin biotin visualization system reactions compared to some biotin-free polymers.

All the polymeric visualization systems used in this study showed sharper staining without cytoplasm staining when compared to the streptavidin-biotin systems. This may be explained by the fact that the polymers utilize a novel controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates, which are free of biotin. Therefore, the problem of non-specific staining that can appear with streptavidin-biotin visualization systems due to endogenous biotin does not occur. However, streptavidin from the kit and endogenous biotin may bind to each other leading to background and cytoplasm staining in most tissues when streptavidin-biotin visualization systems are used [17]. As an example, the streptavidin-biotin system LSAB+, which showed one of the highest staining intensity among the systems, also shows the highest cytoplasm staining among all of them. That applies unreliability to the reaction. Certainly, adjustments could be made in order to reduce or eliminate cytoplasm staining seen in LSAB+, such as reducing primary antibody dilution associated with increased incubation time.

According to some authors, besides allowing cheaper immunohistochemical assays, the high dilutions of the primary antibodies achieved by the systems shown to present stronger staining intensity also enable more reliability to the results. High dilutions prevent background and cytoplasm staining, formation of electrostatic or other non-immunological non-specific bonds, or unexpected cross reactivities (specially with polyclonal primary antibodies) [18].

Last but not least, an additional advantage of the polymeric systems would be the reduction of the staff workload and assay time due to the fewer number of steps present in their protocol.

In the lights of these results, the kits of biotin-free polymeric visualization systems provide sharper immunohistochemical signal without cytoplasm staining when compared to the streptavidin-biotin-system kits. They present variable staining intensity among them due to their differences in molecule structures, reagent types and concentrations, and buffers supplied in the kit. Advance and Novolink were seen to present the highest staining intensity among the biotin-free systems and, followed by the other polymers of second generation, represent a powerful tool for clinical testing and research work. They can contribute to immunohistochemistry standardization of estrogen receptor evaluation of breast cancer.

Competing interests

The authors declare that they have no competing interests'. The authors have no relationship with the manufacturers of the visualization systems cited in the manuscript.

Acknowledgements

We are grateful to the financial support provided by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeicoamento de Pessoal de Nível Superior (CAPES), Brazil.

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BIOTIN-FREE SYSTEMS PROVIDE STRONGER IMMUNOHISTOCHEMICAL SIGNAL IN ESTROGEN RECEPTOR EVALUATION OF BREAST CANCER

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Key words: Breast cancer, Immunohistochemistry, visualization systems, estrogen receptor, digital microscopy

Abstract

Aims: Biotin-free polymeric visualization systems (BFPS) were compared to streptavidin-biotin systems (SABS) in the evaluation of immunoreactivity for estrogen receptor (ER) in breast carcinomas. **Methods:** The anti-estrogen antibody clone SP1 was employed on a tissue microarray containing 320 breast carcinomas. Eleven different detection systems were used: six BFPS of second generation (Advance, Novolink, SuperPicTure, PicTure Max, Super Sensitive Non-Biotin HRP, and Mouse/Rabbit Polydetector HRP/DAB), one BFPS of first generation (EnVision+), and four SABS (LSAB+, EasyPath, Super Sensitive, and Mouse/Rabbit Immunodetector HRP/DAB). The slides were digitalized using the Mirax scanner and the resulting images were analyzed both by an automated method and by visual analysis using the Allred´s score system considering positive nuclear staining. Cytoplasm staining was also separeted evaluated. **Results:** The BFPS Advance and Novolink showed the highest scores by visual analysis, and additionally detected two positive cases which were considered negative using the other detection systems. Likewise, these systems, together with the SAB LSAB+, showed higher staining intensity by the automated method. BFPS revealed no cytoplasm staining, in opposition to the SABS. **Conclusions:** The second generation BFPS, especially Advance and Novolink, provide stronger and sharper nuclear immunohistochemical signal as compared to most SABS, without nonspecific cytoplasm staining. As in few instances these detection systems show discordant results in relation to SABS, further studies correlating these findings to therapeutic response are necessary. BFPS may

represent a high quality tool both for research and clinical evalutation of estrogen receptor in breast cancer.

Copyright Transfer statement:

Competing Interest

None to declare

Introduction

The broadly used streptavidin-biotin visualization systems (SABS) are based on the sequential application of a biotinylated link antibody to streptavidin conjugated to one or two molecules of alkaline phosphatase or horseradish peroxidase (HRP). These conventional biotin-rich procedures profit from the high chemical affinity between streptavidin and biotin. On the other hand, variable nonspecific cytoplasm staining may be detected, especially in biotin rich tissues, as liver and kidney. Recently, new generation of polymeric biotin-free visualization systems (BFPS) have been commercially released. BFPS are based on the polymeric technology, using a compact polymer backbone linked to the enzyme (e. g., HRP) and to an antibody.[1, 2] These polymeric visualization systems are claimed to achieve signal amplification and thereby an enhanced sensitivity by increasing the number of enzyme molecules, which are conjugated to the secondary antibody. Additionally, endogenous biotin does not represent a drawback, as in SABS, and there is a reduction in incubation steps.[3]

In spite of the importance of the choice of visualization systems, which embody an impact on technical feasibility, sensitivity, and interpretation of immunohistochemical specimens, there are only few reports on the use of biotin-free visualization systems.[1, 4, 5] Since they appear to have significant practical clinical relevance, we have comprehensively investigated their comparative sensitivity to the SABS in the immunohistochemical evaluation of estrogen receptor alfa (ER) in breast cancer.

Materials and Methods

Case Selection and Immunohistochemical Procedures

Three hundred and twenty cases of invasive breast carcinomas, diagnosed between 1990 and 2005, were randomly selected to build one tissue microarray (TMA). Slides containing whole sections of previously tested ER positive and negative breast tumors were included in the batch as external control.[6] The present study was approved by the Ethics Committee of the institutions, and performed according to the standard ethical procedures.

Two cylinders of 1mm were obtained from each tumor to build the TMA (Beecher Instruments™, Sun Prairie, WI, USA). Sequentially, 4μm thick sections of the TMA were submitted to immunohistochemical detection of ER using the rabbit monoclonal antibody SP1 (diluted at 1:300; Roche™, Tucson, Arizona, USA). Eleven different visualization systems were used, including six biotin-free polymer systems of second generation (Advance, DAKO™; Novolink, Leica™; SuperPicTure, Zymed™; PicTure Max, Zymed™; Super Sensitive Non-Biotin HRP, Biogenex™ and Mouse/Rabbit Polydetector HRP/DAB, CellMarque™), one biotin-free polymeric system of first generation (EnVision+ DAKO™), and four streptavidin-biotin systems (LSAB+, DAKO™; EasyPath, Signet™; Super Sensitive, Biogenex™ and Mouse/Rabbit Immunodetector HRP/DAB, CellMarque™). Specifications of the visualization systems used in the present study are shown on Table 1. All slides were incubated with the peroxidase block supplied by the manufacturers of each visualization system for 5 minutes. All

antigen retrieval reactions were performed in steamer, using citrate buffer pH=6.0 for 25 minutes. The incubation time was 30 minutes for the primary antibody and also for all reagents of each visualization system. Chromogen (DAB) supplied by each manufacturer was applied for 5 minutes and counterstaining (Harrys hematoxilin) for 2 seconds. All immunohistochemistry reactions were performed simultaneously in order to avoid any bias in the results due to the order of testing or differences in environmental conditions.

Table 1: Specification, type, and supplier of each visualization system used in the present study

Visualization System	Type	Supplier
EnVision+	Biotin-free polymer 1st generation	Dako, Carpinteria, CA, USA
Advance	Biotin-free polymer 2nd generation	Dako
NovoLink	Biotin-free polymer 2nd generation	Leica, New Castle, UK
SuperPicTure	Biotin-free polymer 2nd generation	Zymed, San Francisco, CA, USA
PicTure Max	Biotin-free polymer 2nd generation	Zymed
Super Sensitive non-biotin HRP	Biotin-free polymer 2nd generation	Biogenex, San Ramon, CA, USA
Mouse/Rabbit Polydetector HRP/DAB	Biotin-free polymer 2nd generation	CellMarque, Rocklin, CA, USA
LSAB +	Streptavidin-biotin based system	Dako
EasyPath	Streptavidin-biotin based system	Signet, Dedham, MA, USA
Super Sensitive	Streptavidin-biotin based system	Biogenex
Mouse/Rabbit Immunodetector HRP/DAB	Streptavidin-biotin based system	CellMarque

HRP: horseradish peroxidase; DAB: diaminobenzidine; LSAB: labeled streptavidin-biotin system

Immunostaining Analysis

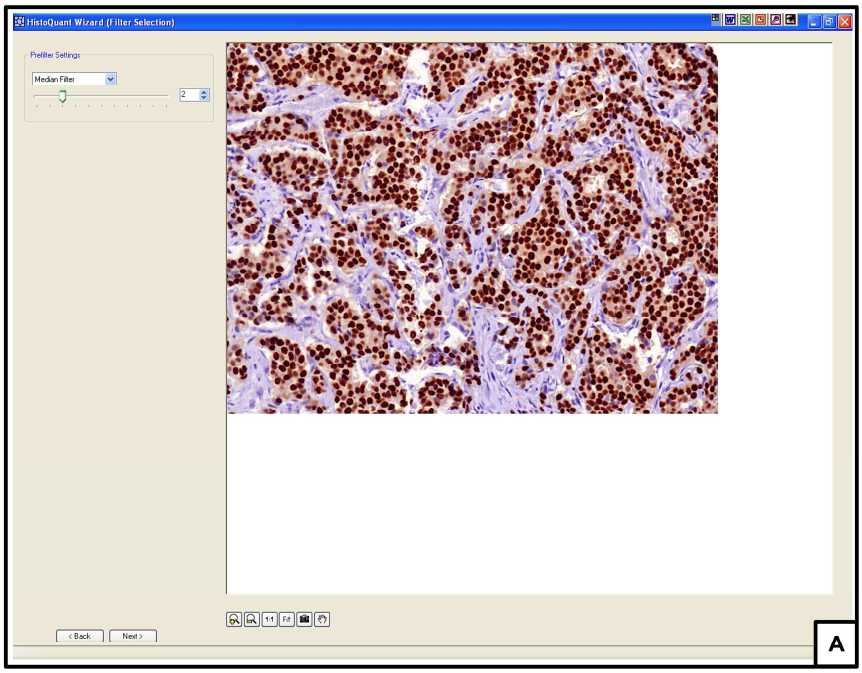
All slides submitted to immunohistochemistry were labeled for blinded examination concerning the visualization system used. All slides were digitalized using the Mirax Scan (Carl Zeiss™, Jena, Germany) and the images provided by the software were exhibited on a LCD monitor under standardized contrast, focus, saturation, and white balance. Both automated image quantification and the visual scoring were made using the images obtained.

To evaluate the staining intensity, the image analysis system HistoQuant™ (3DHistech™, Budapest, Hungary) was used. This software identifies the immunohistochemical staining to be quantified by minimizing background-staining artifacts through image filters. Since the software recognizes the positive nuclei staining of all different intensities, the quantification was processed in each TMA spot automatically by the software.[7-9] Numerical data of staining intensity average corresponding to each spot were exported to a Microsoft Excel file (Figure 1).

For the visual evaluation of positivity, a TMA project was programmed according to the Allred scoring system.[10] Briefly, this system consists of correlating intensity of staining and number of stained cells for the immunohistochemistry evaluation of estrogen receptor in breast cancer, considering negative scores 0 and 2, and positive scores from 3 to 8. The TMA project exhibits each spot at a time on the screen for evaluation together with the

scoring scheme bar shown in the screen footnote (Figure 2). The visual evaluation was performed by a biomedical researcher (RMR, UK-NEQAS assessor) and 20% of all spots were double checked by a breast pathologist (HG) resulting in high agreement of scoring. All data resulting from this evaluation were exported to an Excel file and the agreement with the automated quantification analysis was verified statistically.

Cytoplasm staining was also visually evaluated as absent (0), weak (1), moderate (2), and strong (3) using the TMA project.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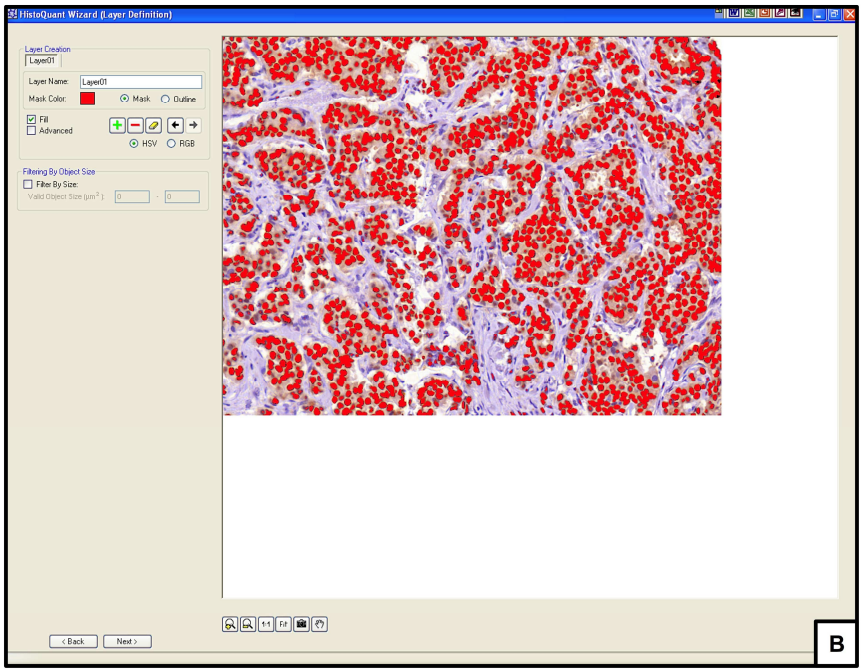
HistoQuant Wizard (Layer Definition)
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Layer Name:
Layer01
Mask Color:
Mask
Outline
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HSV
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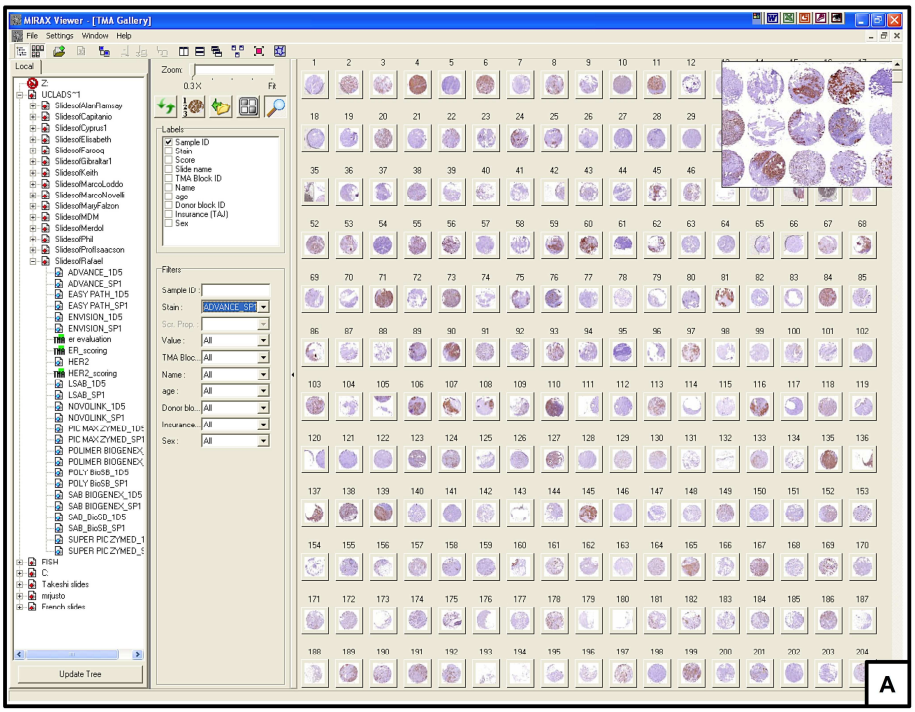
B

Figure 1: Caption of TMA spot from a scanned virtual slide observed in HistoQuant Software for staining intensity quantification at 400x. The primary antibody SP1 and Advance visualization system were employed in this slide. A) Image ready for positive nuclei recognition; B) Positive nuclei already recognized and quantification of immunohistochemical staining intensity being processed.


MIRAX Viewer - [TMA Gallery]
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Local
Update Tree
A

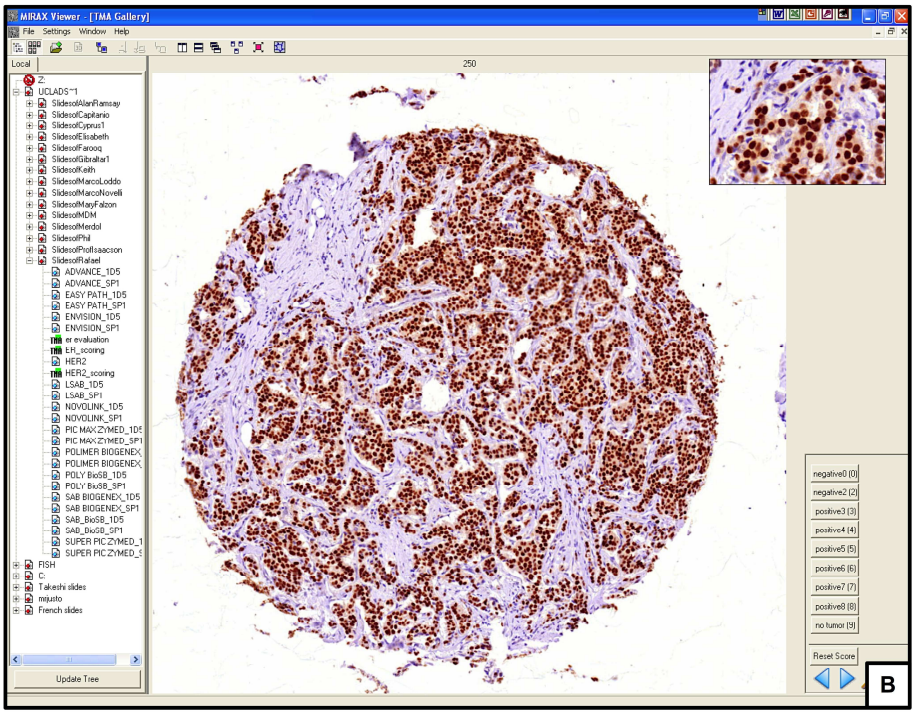
MIRAX Viewer - [TMA Gallery]
File Settings Window Help
Local
Update Tree
negative0 (0)
negative2 (2)
positive3 (3)
positive4 (4)
positive5 (5)
positive6 (6)
positive7 (7)
positive8 (8)
no tumor (9)
Reset Score
B

Figure 2: Virtual slide processed in the TMA Project built in the Mirax Viewer Software. The primary antibody SP1 and Novolink visualization system were employed in this slide. A) 320 numerated TMA spots shown individualized on the screen. B) One single TMA spot ready to be scored according to the Allred scoring system (picture footnote).

Statistical Analysis

The Software WINKS – Statistical Data Analysis, Version 6.0, was employed. The Wilcoxon test was used to compare the different groups of paired parametric and nonparametric variables. The Chi-Square test was applied to evaluate the difference in frequencies of scored values among the groups. Correlation coefficient of Pearson was used to verify the correlation between the different types of analyses.

Results

Taken together, median age of patients was 55y (range 32-77y). 115/320 patients were pre-menopausal and 205/320 menopausal. Stage of disease was I in 202 patients, II in 91 and III in 27. Tumor size presented a median of 12 mm (range 2-50 mm). All cases corresponded to the ductal type. Histological grade was I in 125 patients, II in 163 and III in 32.

According to the automated quantification analysis, the BFPS Advance and Novolink showed the strongest staining intensity together with the

streptavidin-biotin LSAB+. The latter showed stronger staining intensity among all SABS and also among most BFPS. The BFPS PicTure Max and Super Sensitive Non-Biotin-HRP showed intermediate staining intensity, similarly to the SABS Super Sensitive. The polymeric systems SuperPicTure and Mouse/Rabbit Polydetector HRP/DAB showed low intermediate staining intensity similarly to the streptavidin-biotin systems Mouse/Rabbit Immunodetector HRP/DAB and EasyPath. The polymer EnVision+ showed the weakest staining intensity among all the visualization systems. All values obtained by automated quantifying the intensity staining of the different visualization systems and the ***p*** values are summarized on Table 2. Examples of staining from all visualization systems can be seen in Figure 3.

The visual scoring showed high correlation with the automated quantification ($r = 9.1$). When evaluating positivity, the BFPS Advance and Novolink showed 2 positive cases (3+), which had been considered negative when evaluated using the other visualization systems (0 or 1+). We could also separate the visualization systems in three different groups according to the similarities of scoring values achieved by each system evaluated statistically. The first one includes the BFPS Advance and Novolink, which showed the highest scores ($p<0.01$) together with LSAB+ (not included in this group for showing also high level of cytoplasm staining). The second group includes the SuperPicTure, Mouse/Rabbit Polydetector, Super Sensitive non-biotin HRP and PicTure Max, which showed similar scores and there were no discrepancies in positivity among them ($p<0.05$). The third group includes all SABS, which showed higher

cytoplasm staining compared to all BFPS ($p<0.01$) and, apart from LSAB+, showed similar scores among each other ($p<0.05$). LSAB+ showed the highest scores among the SABS ($p<0.01$) and also higher than the BFPS included in the second group ($p<0.05$). However, it also showed the highest cytoplasm staining among all systems ($p<0.01$). The EnVision+ system provided the weakest staining intensity among all systems ($p<0.01$).

The BFPS showed a sharper nuclear staining with no cytoplasm staining, when compared to the SABS. The biotin-free PicTure MAX showed the least cytoplasm staining ($p<0.05$).

Table 2: Comparison of eleven different visualization systems grouped according to their staining intensities *

Staining intensity level	**Biotin-free polymer system**	**Streptavidin-biotin systems**
Stronger	Advance (49,55 ± 16,69; p = 0.0034) NovoLink (43,8 ± 10,17; p = 0.0061)	LSAB+ (45,3 ± 8,98; p = 0.03)
Up intermediate	Super Sensitive non-biotin HRP (39,5 ± 9,34; p = 0.01) PicTure Max (34,8 ± 13,37; p = 0.01)	Super Sensitive (37,7 ± 13,81; p = 0.02)
Low intermediate	SuperPicTure (31,6 ± 10,1; p = 0.01) Mouse/Rabbit Polydetector (31,2 ± 5,89; p = 0.01)	Mouse/Rabbit immunodetector (31,7 ± 7,1) EasyPath (31,8 ± 11,67)
Weaker	EnVision+ (29,5 ± 5,43)	---------

* The mean intensity values among all cases for each visualization system, standard deviation and p values are referred in parenthesis; ***p*** value expresses the statistical analysis between the staining intensity of each visualization system and the group of systems from the level below. There was no significant difference between the staining intensity of the polymeric systems and the

streptavidin-biotin systems of the same level, neither between two systems of the same level group.

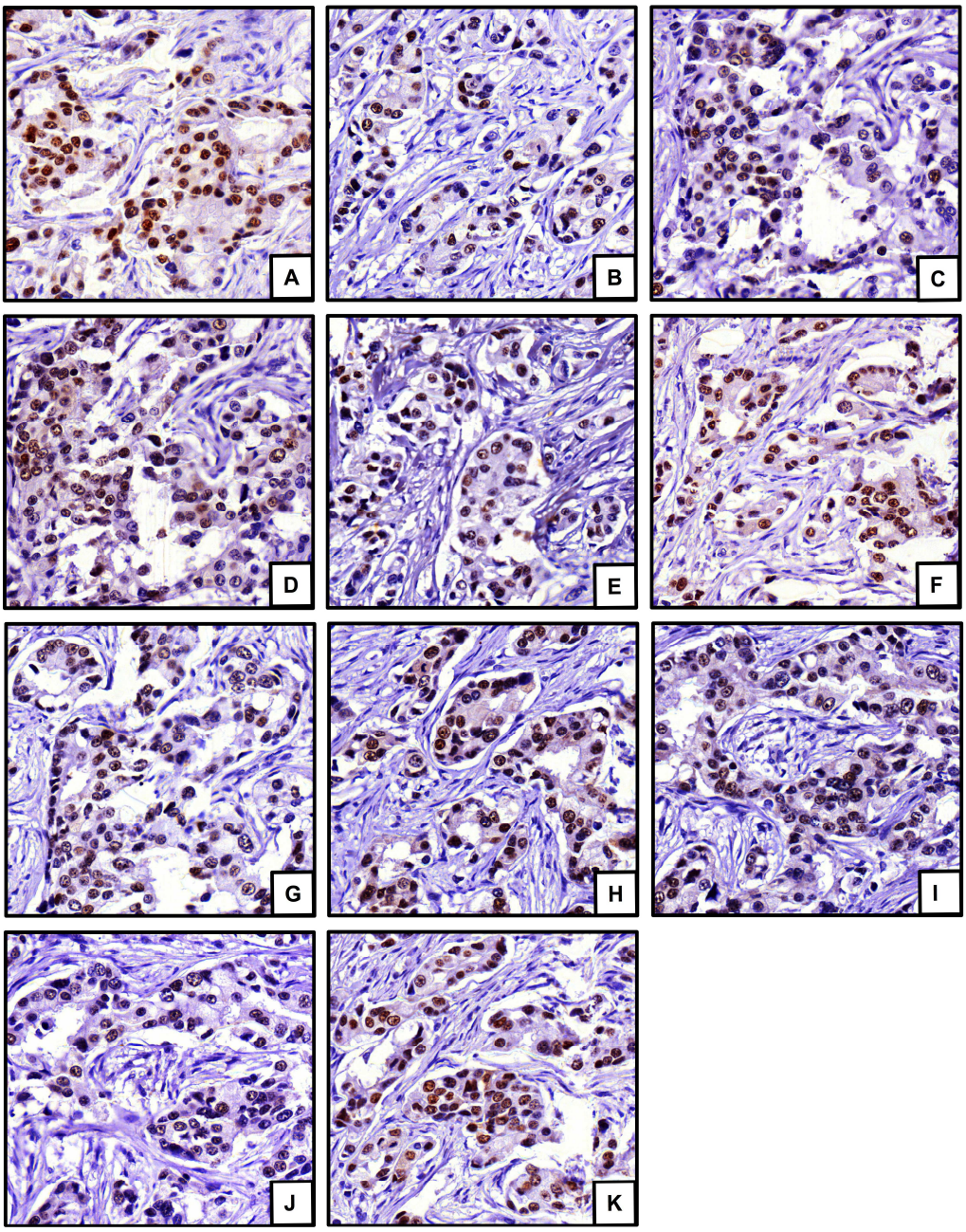

Figure 3: Intensity of immunohistochemical staining in the same tumor using 11 different visualization systems and the rabbit monoclonal primary antibody SP1observed in the objective of 40x: A) Advance, B) EasyPath, C) EnVision, D) LSAB+, E) Novolink, F) PicTure Max, G) Super Sensitive non-biotin HRP, H) Mouse/Rabbit Polydetector HRP/DAB, I) Super Sensitive, J) Mouse/Rabbit Immunodetector HRP/DAB, K) SuperPicTure.

Discussion

Previous studies have compared various commercially available primary antibodies against ER, PR, and HER2, the most clinically relevant predictive markers used in breast cancer.[5, 11-14] Our group itself has carried out a comparison study between different mouse and rabbit ER and HER2 antibodies including the most widely used and recommended by external quality control programs.[13, 14] According to these previous results, in addition to other author's results,[11] we were able to select SP1 as a reliable antibody for the present study. On the other hand, few studies have been carried out to compare different types of visualization systems in immunohistochemistry.[4, 5] In the present study, we have compared the novel BFPS with the conventional SABS to evaluate ER immunohistochemical status in breast carcinomas.

All SABS supply a biotinylated secondary antibody against primary rabbit and mouse IgG and all BFPS also supply a secondary antibody against primary rabbit and mouse IgG, however it is bound to their biotin-free dextran backbone, except by Advance and PicTure Max, which supply a free secondary link

antibody against primary mouse and rabbit IgG. In their case, the polymer will bind this secondary antibody via tertiary antibody bound to the backbone.

According to our results, the BFPS Advance and Novolink showed the strongest staining intensity. These ready-to-use, peroxidase-based visualization systems are compatible with suitably diluted rabbit and mouse primary antibodies. According to the manufacturer, Advance consists of two main reagents: a HRP-Link, which contains anti-mouse and anti-rabbit secondary antibodies in a Tris-HCl buffer, and a HRP-Enzyme, which contains antibodies polymerized with horseradish peroxidase in a Tris-HCl buffer. All BFPS employed in this study do not supply a link antibody, which connects the primary antibody to the antibody bound to the dextran backbone of the polymer, except for the Advance and PicTure Max. This might be one reason for the increase of staining intensity observed in this system. Also, the main characteristic of the second-generation polymers, having an increased number of peroxidase molecules bound to an activated dextran backbone without major gaps among them, might improve chromogen reaction and increase staining intensity.[5] In addition, the first generation polymer EnVision+, which does not present a link antibody and yet shows larger gaps among its peroxidase molecules bound to the dextran backbone, showed the lowest staining intensity among all detection systems used here. Additionally, other reagents supplied in some polymer kits may be important in increasing the staining intensity. The polymer Novolink contains increased number of peroxidase molecules bound to an activated dextran backbone, lacking major gaps among them, in order to improve chromogen

reaction. That might contribute for the increased staining intensity in this visualization system. Novolink also supplies the Post Primary Block, which is used to enhance penetration of the subsequent polymer reagent and might be another reason for its high performance.

In our study, the superior nuclei staining intensity observed with the polymers, as compared to those stained with most SABS, might be explained by the fact that the conventional biotin-rich visualization systems provide a secondary antibody to which fewer enzyme molecules are bond. Instead, the polymeric systems may achieve signal amplification by increasing the number of enzyme molecules conjugated to the secondary antibody through a dextran backbone.[3, 15, 16]

According to our results, the Advance and Novolink received the greatest scores among all the visualization systems, together with LSAB+, and also showed 2 positive cases (3+), which were scored negative (0 and 2+, which is not clinically significant according to Allred [10]) using the other detection systems. Since the anti-hormone therapy is dependent on ER positive result by immunohistochemistry [17-19], the choice of the most sensitive and reliable combination between the detecting primary antibody and the visualization system happen to be of crucial importance according to these findings. However, the strong staining achieved by some visualization systems does not necessarily mean that this system is the best one. Further validation studies correlating these findings to therapeutic response need to be done in order to determine the most suitable combination between the primary antibody and its visualization system.

Of note, a high concordance rate was achieved between the automated and the Allred's visual evaluations, a fact that further supports the clinical feasibility of the latter. It is not possible to assure that those two positive cases in which the visualization system were Advance and Novolink did not represent false positive nuclear staining. This issue should be more precisely addressed by comparing immunohistochemical reactions to gene expression assays, which are not feasible in the present retrospective study. However, we tried to achieve the most reliable reactions by performing negative controls, which were placed in the TMA and also in external slides. Furthermore, non specific reactions occur mostly also in the cytoplasm of cells, a feature that was not observed in our two cases and negative controls.

All polymeric visualization systems used in our study provided sharper staining intensity without cytoplasm staining. This may be explained by the fact that the polymers utilize a novel controlled technology to prepare polymeric HRP-linker antibody conjugates, which are free of biotin. Therefore, the problem of nonspecific staining that can appear with SABS due to endogenous biotin does not occur. In opposition to that, streptavidin from the kit and endogenous biotin may bind to each other leading to background and cytoplasm staining in most tissues when SABS are used.[4, 5, 20, 21]

The high dilutions of the primary antibodies achieved by the polymers also enable more reliability to the results, since very high dilutions prevent background and cytoplasm staining, formation of electrostatic or other non-immunological non-specific bonds, or unexpected cross reactivities (specially

with polyclonal primary antibodies).[4, 13, 22] An additional advantage of the polymeric systems should be the reduction of the staff workload and assay time due to the smaller number of steps needed for the procedure.

In light of these results, the BFPS second generation provide stronger immunohistochemical signal when compared to most SABS. In addition, the BFPS present an unambiguous lack of cytoplasm staining, allowing regarding these systems as powerful tools both for daily clinical routine and for research work. The BFPS may contribute to a more accurate standardization of immunohistochemical evaluation of estrogen receptor status in breast cancer.

Competing interests

The authors declare that they have no competing interests. The authors have no financial or political relationship with the manufacturers of the visualization systems used in the study.

Funding

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Brazil.

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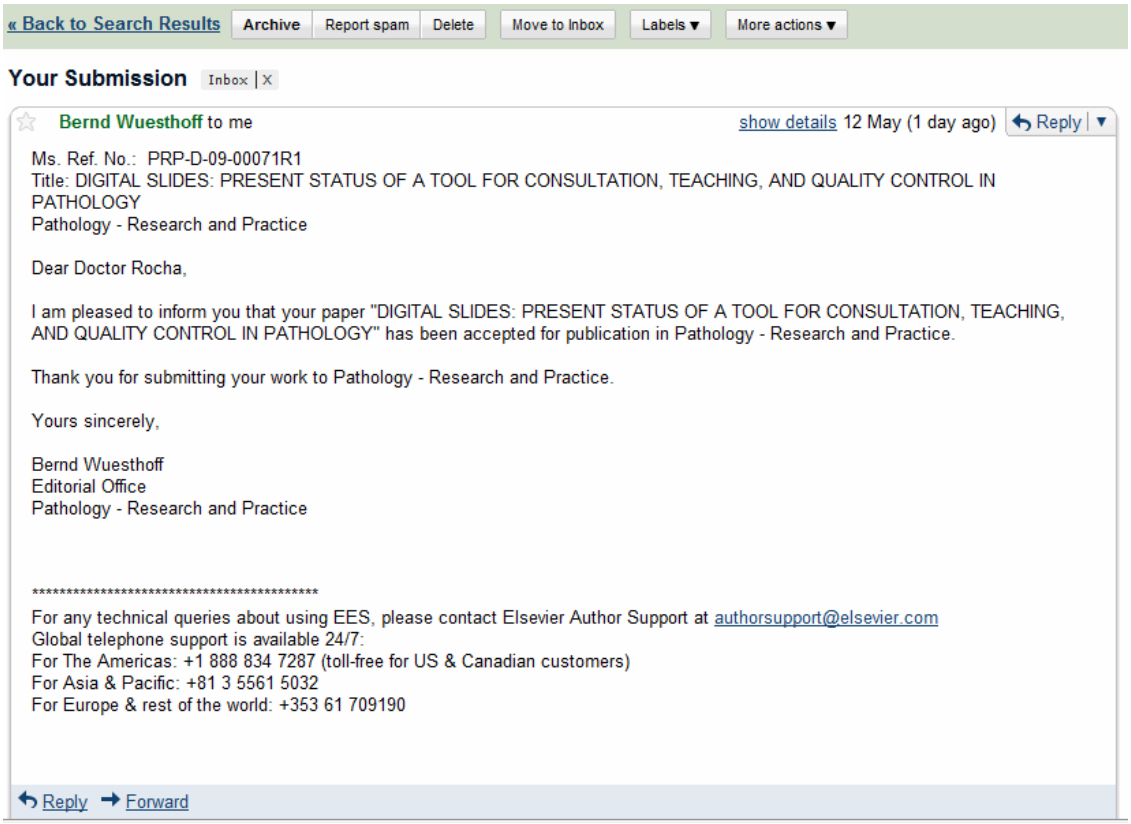

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DIGITAL SLIDES: PRESENT STATUS OF A TOOL FOR CONSULTATION, TEACHING, AND QUALITY CONTROL IN PATHOLOGY

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KEY WORDS: telepathology, review, surgical pathology, teaching, morphometry

ABSTRACT

In the last years, telepathology has benefited from the progress in the technology of image digitalization and transmission through the world web. The applications of telepathology and virtual imaging are more current in research and morphology teaching. In surgical pathology daily practice this technology has still limits, and is more often used for case consultation. In the present review, it is intended to discuss its applications and challenges for pathologists and scientists. Much of the limitations of virtual imaging for the surgical pathologist reside in the capacity of storage of images, which so far hinders the more widespread use of this technology. Overcoming this major drawback may revolutionize surgical pathologist's activity and slide storing.

INTRODUCTION

Despite numerous advances in the understanding of disease processes, most basic aspects of Anatomic Pathology have changed relatively modestly over time. The pathologist supervises the gross dissection of the tissue, which is fixed, dehydrated in organic solvents, embedded in paraffin, sectioned and stained. The tissue specimen is typically examined directly using a light microscope, then the pathologist renders a diagnosis, and the glass slide and paraffin blocks are inevitably relegated to some cumbersome archive. A similar system is employed for Cytopathology, a method which presents the additional problem of being mostly irreplaceable. Thus, the distribution of material for consultative, investigative or teaching purposes remains laborious, and, to a large extent, pathologists only have access to locally available case material for comparison in difficult cases [52].

With the introduction of *in situ* molecular methods in daily practice, especially immunohistochemistry in the 1970s, this scene has begun to change. The year 2000 marked a turning point, at which pathologists began to spend less time in front of light microscopes rendering diagnoses and more time multitasking in front of computers. Although pathologists and their microscopes have been a hallmark of quality medical care for more than a century, the march of time and supervening technologies may alter the way Pathology is practiced in the years ahead [47]. Telepathology surely represents one of such technologies.

The term “telepathology” was first used in 1986, in an editorial of a journal of Pathology [42]. It was followed shortly thereafter by a report in another journal foreseeing in which way telepathology should be used to create the diagnostic networks that were implemented a decade later [7, 9]. Over the past 15 years, there has been increasing interest in technologies that make it possible to examine specimens at a distance.

Up to the 90s, there used to be two forms of telepathology imaging: static and dynamic [11, 33, 43, 46]. In static-image, the referring pathologist captured a small set of digital images that were transmitted and visualized at real time. The dynamic form of telepathology could be carried out by a remotely controlled real microscope. The remote consultant pathologist was able to control the microscope stage and to select the images to be viewed. Some microscopes provided the functionality for selecting various color filters or applying different illumination modes. They also allowed the simultaneous viewing of a slide by multiple clients, although only one client could control the microscope [52].

The new era of telepathology has brought a novel technology concept: the digital slides. Digital slides are virtual entire microscope specimens that can be viewed on a computer screen. They can be accessed from the computer’s hard disk, transportable media (CD-ROM, DVD or memory sticks), or, more practically, from a network server used for image archiving and distribution. [16, 22, 28, 23, 6, 19]. Digital images of adjacent fields in cyto- and histopathological slides are assembled together to form a giant image montage, using a computer controlled scanning stage. Although this technology has existed for more than a decade,

this process, also called whole-slide digitization, has been commercially available for standard desktop computing only since 1998, when sufficient memory and processing speed became available. As the user can explore the entire microscopic section, the digital slides substitute with advantages the real ones in educational venues, remote consultation and may replace cabinets full of slides [23]. Retrieving a slide becomes just a matter of accessing the proper database [38]. It can provide simultaneous access to the slides by multiple users, away from each other, who can access and individually manipulate the same slide or different slides at the same time. In addition, new software modules can be added to perform various types of further processing, as three dimensional image reconstruction from data found in multiple focal planes and on multiple microscope slides, image segmentation and pattern recognition to better characterize known malignancies, and content-based image retrieval, to find all slides with features similar to those in a sample slide [5, 15, 16]. Digital slides can also be used in interlaboratory quality assurance programs. Technical solutions for a web based digital slide viewing system also have been presented, and some examples are available for public evaluation [16, 20, 21, 22, 28, 48, 49].

In the present review, it is intended to present the applications and challenges for pathologists and scientists with the advent of this new technology.

WEB BASED VIRTUAL MICROSCOPY

A digital slide system for public web access is composed of 3 parts: a digital slide acquisition system that acquires the images, a digital slide server that makes the acquired images available on the web, and finally a digital slide client that enables the user to browse the digital slide. The maximum quality of a digital slide is defined by the following factors:

1. Quality of the section.
2. Completeness: the real slide should be accessible as a whole.
3. Image quality: sharpness, contrast, colors, and other attributes of the digital slide should not rank behind those of a real microscope.
4. Usability, for example, smoothly scrolling images, short access times, orientation, several options of magnification.

All these factors are weighted differently by each pathologist. Quality is satisfactory if the pathologist is able to make a correct diagnosis or if the image quality is identical to a real microscope image [28]. In a survey carried out by the Armed Forces Institute of Pathology (AFIP) on 1250 electronic consultations it was shown that major problems were represented by inadequate focus and resolution of images [8]. Evidently, the quality of the section itself finally determines the quality of the resulting digital slide [28]. Using a web-based zoom viewer, digital slides can be magnified from 0.625X to 40X and moved in an *x*–*y* axis on the computer screen, thus emulating the examination of a glass slide with a traditional microscope [12, 13, 18, 50, 22, 44].

Some examples of web-based virtual microscopy are: http://patho.med.uni-magdeburg.de, www.3dhistech.com, www.pathonet.org, www.uiowa.edu, and www.uscap.org.

DIGITAL SLIDE BASED ROUTINE SURGICAL PATHOLOGY – FICTION OR REALITY?

In his review article, O'Brien stated that the computerization of the histology laboratory was desirable but it would be a reality just far in the future [34]. In 2001, some authors already affirmed that complete automated slide digitization had influenced all levels of clinical practice and education, and they also emphasized the importance of dedicated software technology [22]. More recently, digital slides and digital microscopy technology have already been used in selected cases for telepathology consultation. It is reasonable to foresee that, after the definition of time and storage requirements, the routine use of this technique can also be considered [6]. Up to date, some issues need to be developed in order to apply the digital slide technology to clinical practice in a more efficient way, such as:

1. Development of new user-friendly software suitable to the routine workflow in a faster and practical way.
2. Development of software, which can be linked to the laboratory database.
3. Improvement in scanning speed.

4. Improvement of the computers' or external servers' capacity for data storage.

5. Development of economical scanners and software.

The convenience of quickly analyzing conventional slides on conventional microscopes by the pathologists may cause resistance against such a new technology. The current high cost of the scanners associated with deficiencies in non user-friendly software may be an important reason for the delay in broadly validating the scanning systems. Also, the process of scanning the material is a significant additional cost of time and labor to the traditional processing costs. Recently, the Aperio scanning system and its image analysis software, one of the commercially available equipments, received the approval of the U. S. Food and Drug Administration (FDA) for digital evaluation of HER2 and hormone receptors by immunohistochemistry (www.fda.gov). In spite of the above cited necessary improvements and further validation studies, this fact represents an important shift from research to efficient clinical practice employing digital slides.

DIGITAL SLIDES FOR PATHOLOGY EDUCATION

Traditional glass slides and standard microscopes have been used in pathology education for many decades. However, this has been problematic, because stains fade over time, they get damaged, are sometimes difficult to duplicate, may be hard to distribute, and the quality of microscopes provided for educational purpose is often poor. Early in the last century, photomicroscopy

enabled the pathologist to avoid some of these pitfalls and to extend the availability of microscopic observations in the form of traditional black and white photographs and 35 mm slides. The dynamic interaction between the viewer and the glass slide, however, is severely limited by the use of static images. Viewing static images does not faithfully reproduce the intellectual process through which information is gathered and analyzed from the perceptual evaluation of the entire information contained in the glass slide. It cannot substitute the process of visually scanning the image at varying magnifications or comparing different areas of the glass slide to reach a final impression of the pathologic process. With the advent of digitized images during the past few decades, static digital images on CD-ROMs or on the internet have begun to replace traditional photographs in pathology education, without the disadvantages of glass slides. Recent advances in digital microscopy have permitted the generation of very large images that can be captured by robotic photomicroscopes to create “digital” slides. The digital slide is often so large that a special viewing application, referred to as a virtual microscope, is required [17].

Even not using digital slides, web-based educational methods have been developed for pathology, cytology, surgery, nursing, radiology, emergency medicine, and anatomy, allowing continued medical education, patient information, and self-assessment in these subjects [1, 3, 4, 24, 29, 30, 31, 32, 35, 36, 37, 39, 45, 53, 54]. Steinberg et al [53] reported their experience at the Johns Hopkins School of Medicine with an interactive case- and web-based approach to teach cytopathology to second-year medical students. Preliminary

results were encouraging, as the students have enjoyed the experience and increased their knowledge on the matter. Kronz et al developed another web-based tutorial for practicing pathologists that has been very effective in improving practitioners' ability to provide Gleason grading to images of needle biopsy prostate carcinoma specimens [26]. With the same purpose, a digital atlas of breast histopathology has been published (www.webmicroscope.net/breastatlas) [32]. Web-based image technology has also allowed to assess inter-observer agreement of diagnosis [2, 25].

Starting in 1998, the course on Pathophysiology of Disease at UCLA's School of Medicine was reorganized to take advantage of rapidly developing web-based technologies permitting more efficient distribution of standardized teaching materials to a large group of students. The "individual microscopic sessions" of the pulmonary pathophysiology course were replaced by self-instructional, interactive laboratory sessions using web-based technology and case-based instruction. These sessions replaced traditional microscopic teaching using multiheaded microscopes and multiple instructors, requiring only one pneumologist and one pathologist, who discuss the cases with the students with great benefit [4].

HISTOPATHOLOGY QUALITY ASSURANCE USING DIGITAL SLIDES

Professional proficiency testing has become an integral part of continuous professional development in all medical specialties in the United Kingdom and North America. Ideally, proficiency should be measured on the same "task"

material for each individual. In the United Kingdom, for example, national quality assurance scheme in breast screening pathology comes close to the ideal practical solution. In this scheme, all consultant histopathologists involved in management of breast disease are confidentially assessed twice a year by examining two sets of 12 microscopic slides selected from the spectrum of breast diseases. Individual consultant's diagnoses on these slides are compared with the consensus diagnosis of 19 experts in breast pathology. A traditional evaluation including more than 400 pathologists would easily take about five months to be completed. The disadvantages are easy to anticipate: circulation of slides by mail to all participants; variability of morphologic representation in the consecutive sections, especially in focal lesions; difficulty in circulating more infrequent processes or interesting lesions detected in small biopsy specimens. For these reason, the use of digital slides represents a powerful tool for the assessment of diagnostic accuracy and quality control programs.

As the quality assurance scheme provided by the College of American Pathologists (CAP) already employ telepathology by supplying images to be evaluated by the pathologists via web (static pathology), the leaders of the United Kingdom National External Quality Assessment Service (UK-NEQAS) have demonstrated interest in digital slides as a tool for receiving the laboratories' stains and to send them to referees residing in different corners of the world.

AUTOMATED IMAGE ANALYSIS APPLIED TO DIGITAL SLIDES

Automated histological analysis has proved to be an important research tool, and, more recently, software capable of identifying, isolating, and quantifying different types of stains has become suitable for routine diagnostic practice.

Our group has carried out a study on immunohistochemical staining intensity in digital slides using commercially available software. We have used the Mirax Scanner™ to digitalize the slides, the software Mirax Viewer™ for viewing and isolating the tissue microarray spots, and the software HistoQuant™ for the automated quantification of staining, all from 3DHistech™ (Budapest, Hungary). We have quantified the immunohistochemical staining for estrogen receptor in breast carcinomas using 11 different types of visualization systems in order to identify the strongest stains. Numerical data of staining intensity average corresponding to each spot obtained by the software was exported to a Microsoft Excel file, and compared to the visual scoring system proposed by Allred [27]. Correlation between both scoring was high (r=9.1), supporting the reliability of the software. However, no cut-off value to consider a case estrogen receptor positive for therapeutic purposes in breast cancer could be defined as yet [40].

So far, no image analysis software has allowed the establishment of cut-off values for immunhistochemical evaluation in routine diagnosis. Moreover, the identification of the target cells to be included in the analysis still represents a challenge. As validation studies on the reliability of each commercially software released are made, the offer of improved and user-friendly image analyzers can

increase, and the employment of automated staining analysis for routine diagnostic can be more widely used and standardized.

COMMERCIALLY AVAILABLE SCANNING EQUIPMENTS

There are different options of commercially available slide scanners (Table 1). More suppliers have shown up, and the suppliers of immunohistochemistry material have been investing in this new technology, offering packages for automation from the reaction to the interpretation of results. Before making a decision on which equipment to purchase, some issues must be considered:

1. The purpose of purchasing the equipment.
2. Number of slides per session acceptable by the machine.
3. Speed of scanning.
4. Quality (resolution) of the image achieved and type of camera.
5. If the equipment also scans immunofluoresce and fluorescence *in situ* hybridization (FISH) slides, if this is a necessity of the laboratory.
6. Facilities of technical assistance provided by the supplier.
7. Software facilities according to the laboratory necessities.

Table 1: Examples of commercially available slide scanners and suppliers.

Equipment	**Supplier**
Acis	DAKO, Carpinteria, CA, USA
Aperio ScanScope	Aperio, Vista, CA, USA
Mirax Scanner	3DHistech, Budapest, Hungary
NanoZoomer	Leeds, Minneapolis, MN, USA

FUTURE CHALLENGES

While hardware for digitizing tissue samples and microscopy slides more effectively are rapidly becoming commercially available, software support required to store, retrieve, and process digitized slides, providing optimal interactive response times, remains a challenging issue. At the basic level, the system should emulate the usual behavior of a conventional microscope, including continuously moving the slide, changing magnification and focus. The processing for viewing a slide requires projecting high resolution data onto a grid of suitable resolution (governed by the desired magnification) and appropriately compositing pixel mapping onto a single grid point, to avoid introducing spurious artifacts into the displayed image. The main difficulty in providing the basic functionality is storing and processing the extremely large quantities of data required to digitalize a large collection of slides [52]. Since the equipments are made to scan the slides at high magnification (20 or 40X) and multiple focal planes in depth, one single digital slide may vary from 3 to 50 gigabytes. Thus,

storage needs are exacerbated by the fact that hospitals can generate many thousands of slides per year. For example, at the Johns Hopkins Hospital (Baltimore, MD, USA) the histology laboratory processes 420 000 routine, special-stain, and immunohistochemical slides per year. Clearly there is an enormous storage requirement. Due to this massive need for storage space, there are also attendant difficulties in achieving rapid response time for various types of inquiries into the slide image database [38, 52].

Such new technology may bring different expectations for the next few years and some human barriers may be important in the consolidation of the universe of digital slides. Difficulties of working with non user friendly software, concerns about how this technology would affect the smaller laboratories and how far it could go on replacing human expertise may be topics to worry about. It is expected that the suppliers provide proper training to the real and potential customers and also quality control program organizers regarding the usage of the hardware and software as well as invest in research for developing new software which are more user friendly and reliable. Also, lectures, conferences and satellite symposia on the subject should be provided aiming to achieve as many people as possible. The idea of receiving a brand new technology may seem chaotic especially for those who are completely used to conventional methods. However, in a gradual, smooth, and continued way, not the people themselves, but the concept of work manner can change in the experts' minds, since technology, such as any type of culture, has got a dynamic characteristic.

According to all this, developments of more efficient software for slide viewing and analysis, information storage capacity, and also dedication from the suppliers in training processes and advertisements might be the greatest challenges for this new technology in the next few years.

TAKE HOME MESSAGES

- Digital slides associated with imaging software are being widely used for research practice in the last three years. Although some systems have received the FDA approval for such practice, its usage for diagnostic routine is still restricted.

- Digital slides have been progressively used for educational purpose throughout the web, and allow identical images in numerous different computer screens, do not get damaged, and do not cause problems for storage or retrieval.

- Digital slides represent a promising tool for quality assurance schemes, avoiding problems and costs related to conventional mail. Assessors will be able to access digital slides from their own office decreasing travel costs and time.

- Software for image analysis can be easily applied to digital slides for research purposes and, more recently, for routine diagnosis.

- Two main challenges can be pointed for the next few years: the development of new software, which should be more user friendly, and

suitable to research and routine needs; and improvement of image storage capacity of hard disks.

ACKNOWLEDGMENTS

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Hospital A. C. Camargo, Brazil.

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DIGITAL SLIDES: A NEW POWERFUL TOOL FOR PATHOLOGY EDUCATION AND QUALITY CONTROL

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GENERAL INFORMATION

The year 2000 may have marked a turning point at which pathologists began to spend less time in front of light microscopes rendering diagnoses and more time multitasking in front of computers. Although pathologists and their microscopes have been a hallmark of quality medical care for more than a century, the march of time and supervening technologies may alter the way pathology is practiced in the years ahead (1). Digital slides represent such technology.

Digital slides are digitized entire microscope specimens that can be viewed on a computer screen. Digital slides can be accessed from the computer's hard disk, transportable media (CD-ROM, DVD or memory sticks), or, more practically, from a network server used for image archiving and distribution (2). Digital slides are acquired by assembling digital images of adjacent fields in histological sections or fluid smears into a giant image montage using a computer controlled scanning stage. Although this technology has existed for more than a decade, this process (also called whole-slide digitization) has been commercially available for standard desktop computing only since 1998, when sufficient memory and processing speed became available. Because digital slides can capture the important visual information of a glass slide, they can be used in all of the educational venues in which glass slides and digital photomicrographs are used, without losing the ability inherent in traditional glass slide microscopy to explore the entire microscopic section, identify structures and lesions, and demonstrate their relationships and variations (3). By simply

emulating the usual behavior of a physical microscope, such a system can replace cabinets full of slides with a digital storage subsystem. Retrieving a slide then becomes a matter of accessing the slide database, without requiring physical access to the slide. One example of additional capability is in a teaching environment, where an entire class of students can access and individually manipulate the same slide at the same time, searching for a particular feature in the digital slide. Another example is remote consultation, where a physician in a different part of the country, or even in another country, can view a slide via internet, once the slide has been scanned and placed in an accessible database (4). It can provide simultaneous access to the slides by multiple users, who can access and individually manipulate the same slide or different slides at the same time as in a real microscope.

The world of digital slides and telepathology will be discussed along this review including its appliance for didactic purposes and quality assurance schemes as well as the challenges for pathologists and scientists with the advent of this new technology.

DIGITAL SLIDES FOR PATHOLOGY EDUCATION

Traditional glass slides and standard microscopes have been used in pathology education for many decades. However, the use of glass slides in education has been problematic, because they break, are difficult to duplicate, fade over time, and are difficult to distribute. Early in the last century, photomicroscopy enabled the pathologist to avoid some of these pitfalls and

extend the availability of microscopic observations in the form of traditional black and white photographs and 35 mm slides. The dynamic interaction between the viewer and the glass slide, however, is severely limited by the use of static images. Viewing static images does not faithfully reproduce the intellectual process through which information is gathered and analyzed from the perceptual evaluation of the entire information contained in the glass slide. It cannot substitute the process of visually scanning the image at varying magnifications or comparing different areas of the glass slide to reach a final impression of the pathologic process. With the advent of digitized images during the past few decades, static digital images on CD-ROMs or on the internet have begun to replace traditional photographs in pathology education because the digital image does not break, is easy to duplicate and distribute, and does not fade. Recent advances in digital microscopy have permitted the generation of very large images that can be captured by robotic photomicroscopes to create “digital” slides. The digital slide is often so large that a special viewing application, referred to as a virtual microscope, is required (5).

Even not using digital slides, web-based educational methods have been developed for pathology, cytology, surgery, nursing, radiology, emergency medicine, anatomy, continuing medical education, patient education, and other related activities. (6) For example, Steinberg et al (6) reported their experience at the Johns Hopkins School of Medicine with an interactive, case- and web-based approach to teaching cytopathology to second-year medical students. Preliminary results from this experience are encouraging; the students have

enjoyed the experience and increased their knowledge about basic cytology. Kronz et al. developed another web-based tutorial for practicing pathologists that has been very effective in improving practitioners' ability to provide Gleason grading to images of prostate carcinoma specimens obtained by needle biopsy (6). Starting in 1998, the Pathophysiology of Disease course at UCLA's School of Medicine was reorganized to take advantage of rapidly developing web-based technologies that allowed for the efficient distribution of standardized teaching materials to a large group of students. The "individual microscopic sessions" of the pulmonary pathophysiology course were replaced by self-instructional, interactive laboratory sessions using web-based technology and case-based instruction that allows the students to study digital images representing selected pathologic conditions at their own place. The microscopic sessions using multiheaded microscopes and multiple instructors were replaced by 2 discussion sessions. Led by a pneumologist and a pathologist, the students discuss the cases available on the website. The cases include a clinical history; description of physical examination and laboratory tests; and pulmonary pathophysiology, imaging, and pathologic information (6). The success of the web-based technology as a teaching method also encouraged these authors to state that it would benefit from the appliance of digital slides to this technology (6).

Up to 4 years ago, image reference systems and Web tutorials were based on conventional microscopy images limited to only one magnification level and a preselected specimen area. Recently, the act of changing magnification when using conventional light microscopy simulated with zoomable images of the

entire microscope slides digitized at a high resolution allows the user to view any part of the specimen at any magnification (7). Another study showed the usage of digital slides to evaluate the Gleason grading system, which is the most widely used grading system for prostatic carcinoma. The study also compared the interobserver variation in applying the grading system (7). They demonstrated that web-based virtual microscopy is well suited for interactive education, which is, for self-testing and learning Gleason grading of prostate carcinoma. They concluded that web-based virtual microscopy is an effective method to evaluate interobserver variability and that it is a promising new tool for teaching and standardizing the use of Gleason grading among training pathologists. The use of these advanced techniques allows transferring image data over the internet. Web-based virtual microscopy has been used as a powerful platform for various educational applications in histopathology (Henrik). The same authors have also published digital atlas of histopathology such as: www.webmicroscope.net/breastatlas (7), http://patho.med.uni-magdeburg.de, www.3dhistech.com, www.pathonet.org, www.uiowa.edu, and www.uscap.org.

HISTOPATHOLOGY QUALITY ASSURANCE USING DIGITAL SLIDES

Professional proficiency testing has become an integral part of continuous professional development in all medical specialties in the United Kingdom and North America. Ideally, proficiency should be measured on the same “task” material for each individual. In the United Kingdom, for example, national quality assurance scheme in breast screening pathology comes close to the ideal

practical solution. In this scheme, all consultant histopathologists involved in management of breast disease are confidentially assessed twice a year by examining two sets of 12 microscopic slides selected from the spectrum of breast diseases. Individual consultant's diagnoses on these slides are compared with the consensus diagnosis of 19 histopathologists regarded as expert in breast diseases' diagnosis. However, with more than 400 pathologists participating in the scheme, having to circulate slides by mail to all participants results in each proficiency round taking 5 months to complete. The sections, although consecutive, are not identical, yet individuals are judged on what is assumed to be present in the section rather than its actuality. Presenting the same microscopic data electronically to all participating pathologists could eliminate deficiencies. So, the use of digital slides came as a promising tool for investigating the accuracy of diagnosis and utility of telepathology in pathology screening. Beyond providing identical images to be analyzed by all pathologists involved in the program, digital slides also provide time saving on regular mail, lack of risks of broken slides and comfort for analysis.

The quality assurance scheme provided by the College of American Pathologists (CAP) already employ telepathology in its program by supplying images to be evaluated by the pathologists via web (static pathology). The heads of the United Kingdom National External Quality Assessment Service (UK-NEQAS) stated their interest in digital slides as a tool for receiving the associated laboratories' stains and also for assessing them by assessors from different parts of the world.

FUTURE CHALLENGES

While the hardware for digitizing tissue samples and microscopy slides more effectively is rapidly becoming commercially available, the software support required to store, retrieve, and process digitized slides to provide interactive response times for the standard behavior of a physical microscope remains a challenging issue. The basic level, the system should emulate the usual behavior of a physical microscope, including continuously moving the stage and changing magnification and focus. The processing for viewing a slide requires projecting high resolution data onto a grid of suitable resolution (governed by the desired magnification) and appropriately compositing pixels mapping onto a single grid point, to avoid introducing spurious artifacts into the displayed image. The main difficulty in providing the basic functionality is storing and processing the extremely large quantities of data required to represent a large collection of slides (8).

Developments of more efficient software for slide viewing and analysis and also information storage capacity might be the greatest challenges for this new technology in the next few years.

TAKE HOME MESSAGES

- Digital slides associated with imaging software are being widely used for research practice in the last 3 years. Although some systems have

received the FDA approval for such practice, its usage for diagnostic routine is still restricted.

- Digital slides have been progressively used for educational purpose throughout web, and allow identical sections in numerous different computer screens, do not break, and do not cause problems for storage or retrieval.

- Digital slides represent a promising future for the quality assurance schemes avoiding problems and costs related to conventional mail. Assessors will be able to access digital slides from their own office decreasing cost and time.

- Software for image analysis can be easily applied to digital slides for research purpose and, more recently, for routine diagnosis.

- Two main challenges can be pointed for the next few years: the development of new software, which are more user friendly and suitable to research and routine needs; and improvement of the image storage capacity of hard disks.

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Comentários finais

Comentários finais

Os sistemas de visualização livres de biotina de segunda geração mostram marcação imuno-histoquímica mais forte quando comparados aos sistemas biotinilados, com exceção do LSAB+. Os polímeros Advance e Novolink mostraram os sinais mais fortes e ainda apresentaram casos positivos para receptor de estrógeno que foram considerados negativos para todos os outros sistemas de visualização, o que determina mudança na conduta terapêutica da paciente. No entanto, estudos correlacionando estes achados com resposta terapêutica de pacientes necessitam ser feitos para validar estes resultados.

Os polímeros livres de biotina apresentam reações imuno-histoquímica mais limpas e sem marcação citoplasmática quando comparados aos sistemas de estreptavidina-biotina. Isto faz destes novos sistemas uma importante ferramenta para a rotina clínica e para investigação, contribuindo na padronização imuno-histoquímica para a avaliação de receptor de estrógeno em câncer de mama.

Os dois sistemas de análise das reações, visual e automatizada, mostraram semelhança com alta concordância de resultados. A análise visual mostrou as diferenças de positividade entre os sistemas de visualização segundo o sistema de escore adotado, e a análise automatizada complementou a interpretação do observador diminuindo a subjetividade e aumentando a fidedignidade da avaliação de receptor de estrógeno em câncer de mama.

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