Confocal Examination of Untreated Fresh Specimens From Basal Cell Carcinoma

Implications for Microscopically Guided Surgery

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Objective: To evaluate the diagnostic accuracy of confocal examination of basal cell carcinoma (BCC) in microscopy-guided surgery.

Design: Four independent observers with no previous experience in confocal laser scanning (CLS) microscopy received standardized instruction about diagnostic CLS microscopic features. Subsequently, 120 confocal images of fresh excisions from BCCs or normal skin were evaluated by each observer, imaged using a commercially available, near-infrared, reflectance CLS microscope. Logistic regression analysis was performed on a combination of all morphologic features using the forward-stepwise (Wald) method. Reliability (interobserver agreement) data were evaluated by \( \kappa \) statistic.

Setting: Department of Dermatology, Medical University of Graz.

Patients: Twenty patients with histologically verified BCC.

Interventions: Evaluation of fresh BCC excisions by CLS microscopy.

Main Outcome Measures: Diagnostic accuracy of the method was evaluated by \( \chi^2 \) test. Diagnostic impact and reliability of each morphologic feature were evaluated by logistic regression analysis and \( \kappa \) statistic, respectively.

Results: Overall, high diagnostic accuracy was achieved by the 4 observers. Logistic regression analysis revealed that mainly tumor cell nuclei and tumor nests should be taken into account for diagnostic decisions, whereas disintegration of tumor cells, peripheral palisading, and retraction of stroma were rarely useful. However, most of the features were highly reliable.

Conclusions: This diagnostic validation study of CLS microscopy in microscopy-guided surgery yielded promising results and opens avenues for further studies. In the future, CLS microscopy may guide microsurgery of any skin cancer.

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Basal cell carcinoma (BCC) is the most common skin tumor in white patients.\(^1\) In the United States, an estimated 1 million cases of BCC occur every year.\(^2\) An increasing incidence of BCC corresponds with changes in lifestyle and various environmental factors such as ozone depletion and increased exposure to UV radiation.\(^3\) Basal cell carcinoma is associated with considerable cosmetic and functional morbidity. Increasing numbers of younger individuals are affected, and BCC treatment consumes considerable medical resources. Basal cell carcinoma most commonly occurs on the face, head, and neck of people older than 40 years.

Since BCC occurs in high-risk sites such as on or near the nose, eyes, ears, and mouth, precise microsurgical excision must be performed to remove the entire cancerous lesion with minimum damage to the surrounding normal tissue. One such precise technique for treating nonmelanoma skin cancer is Mohs micrographic surgery, guided by the intraoperative histopathologic analysis.\(^4,5\)

See also page 1318

However, in many cases a Mohs procedure requires several excisions to remove large complex lesions. In addition, the preparation of frozen, hematoxylin-eosin–stained sections typically requires 20 to 45 minutes per excision, during which time the patient must wait with an open wound under local anesthesia. This is slow and time inefficient for Mohs surgeons. Finally, Mohs microsurgery

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requires specialized equipment and trained medical and technical personnel that add substantial cost to the treatment and, furthermore, are not always available.

If Mohs micrographic surgery is not available under certain conditions, transoperative histologic examination with delayed closure of the wound can be performed.6,7 A map of the affected area is drawn for each lesion, indicating the exact location of the tumor. Afterwards, the tissue is cut into separately numbered pieces, embedded in paraffin, and stained with hematoxylin–eosin. If margins are found to be positive for tumor, further resections are carried out until the margins are free of tumor tissue. However, this procedure requires up to several days during which the surgical defect must be left open.

An interesting alternative approach for microscopy-guided surgery of nonmelanoma skin cancers was recently introduced by Rajadhyaksha et al,8 who used a confocal reflection microscope to search for residual tumor areas in the excision margins in cases of nonmelanoma cancer. To enhance contrast of nuclei within the BCC, skin excisions were fixed with 5% acetic acid, which causes compaction of chromatin within the nuclei due to extraction of histone proteins.9 In turn, this compaction increases light backscatter and makes the nuclei bright and easily detectable when examined using confocal microscopy. Confocal images compared well with corresponding histopathologic analysis. The location, shape, and size of the tumor islands were clearly delineated. Furthermore, the atypical structure of individual nuclei became evident. Remarkably, the authors reported saving 17 to 36.5 minutes compared with frozen section histologic analysis.8 However, diagnostic accuracy of the method and statistical evaluation of diagnostic morphologic features have not been assessed.

To our knowledge, the present study is the first to systematically validate confocal microscopy in diagnosing BCC in untreated and fresh skin excisions during microscopy-guided surgery. We evaluate the diagnostic impact of the method and analyze diagnostic morphologic features determined by confocal microscopy for their presence or absence, diagnostic performance, and reliability.

METHODS

SURGICAL EXCISIONS

Twenty patients with histologically verified BCC were recruited prospectively from the Dermatologic Surgery Unit at the Department of Dermatology, Medical University of Graz, Graz, Austria. They gave informed consent for surgical excision of their lesions. All institutional rules governing clinical investigation of human subjects were strictly followed. An appropriate institutional review board approved the project. Only tumors with a clinically determined horizontal diameter exceeding 10 mm were included in the study. A 3-mm punch biopsy specimen was taken from the center of the tumors by the surgeon in each case before the whole lesion was excised according to standard protocol and submitted to transoperative histologic examination. Furthermore, normal-appearing skin distant from the tumor bulk was collected from the site of “dog ears” of the spindle-shaped resections. Thus, the experiments did not interfere with the routine surgical and histotechnical procedures and patient care and provided 2 pieces of skin tissue, 1 definitely containing tumor tissue (obtained from the tumor bulk) and 1 definitely free of tumor tissue (obtained from a distant site of the area). From the fresh skin excisions, approximately 0.5-mm-thick vertical sections were prepared, washed with 5% acetic acid for 30 seconds, placed onto a glass slide, and then imaged with a confocal microscope.

CONFOCAL LASER SCANNING MICROSCOPY

Confocal laser scanning (CLS) microscopy was performed with a commercially available, near-infrared, reflectance confocal microscope (Vivascope 1000; Lucid Inc, Rochester, NY). The Vivascope 1000 uses a diode laser at 830-nm wavelength and a power of less than 35 mW at tissue level to avoid all tissue damage. A ×30 magnification, water-immersion (refractive index, 1.33) objective lens with a numerical aperture of 0.9 was used. This device imaged with a spatial resolution of 0.5 to 1.0 µm laterally and 3 to 5 µm in the axial dimension, allowing visualization of cellular structures of the examined specimens.

Usually, an examination depth of 350 µm was reached. The Vivascope 1000 provided a field-of-view of approximately 0.5 × 0.5 mm on tissue level. Individual images of the entire field were captured and saved. Adjacent fields were imaged in sequence and then tiled together to produce a composite image of an area of tissue approximately 2.5 × 2.5 mm.

The objective lens of the microscope was placed onto an adapter ring, which was fixed on a glass slide. Overview images (maps) were captured in a 2-dimensional matrix using the composite image procedure. These maps were used to locate suspected cancerous sites. Within the map, individual images were selected, and the system resumed imaging in that area of the tissue. At least 20 images comprising epidermal and dermal areas and tumor tissue (when present) were recorded in each specimen and stored using the BMP file format.

DIAGNOSTIC MORPHOLOGIC BCC FEATURES

Morphologic CLS microscopic features of BCC were assessed according to standard criteria used in conventional histopathologic analysis.10 In brief, large and oval or elongated tumor cell nuclei (rather uniform, no pronounced variation in size, and with nonepithelial appearance) were taken into account for further analysis, as were tumor nests of various sizes and shapes (elongated or intertwining strands, concentric whorls, tumor cells around cysts or radially arranged around islands of connective tissue, and buds and irregular proliferations of the tumor tissue attached to the undersurface of the epidermis), disintegration of tumor cells in the center of tumor masses (cystic spaces), palisade arrangements of the peripheral cell layer of the tumor masses, and retraction of the stroma from tumor islands resulting in peritumoral lacunae.

TRAINING DATA AND STUDY SET

Four independent observers (2 residents and 2 dermatopathologists) with no previous experience in confocal microscopy received standardized 30-minute oral presentation instruction about morphologic CLS microscopic features of BCC and normal skin. Diagnostic BCC features (tumor cell nuclei, tumor nests, disintegration of tumor cells, palisade arrangement of peripheral tumor cell layer, and retraction of stroma) and morphologic CLS microscopic appearance of normal skin structures (including epidermal appendages) were explained, and 10 image examples each of BCC and normal skin were demonstrated.

For diagnostic assessment, 3 confocal BCC and 3 normal skin images were selected from each of the 20 patients. Sixty image pairs comprising BCC and normal skin from corresponding areas of the specimens were shown on the computer screen in random sequence. Each observer evaluated the image pairs...
and allocated the image that he or she considered to contain tumor tissue. In a second run, the presence or absence of each of the morphologic BCC features was assessed in the study set of 120 confocal images by 1 resident and 1 dermatopathologist. All of the experimenters were blinded as to the clinical and histopathologic diagnosis of the confocal images.

STATISTICAL ANALYSIS

Statistical analysis (χ² test, sensitivity, specificity, κ statistic, and logistic regression) was performed on the data sets with a personal computer using the SPSS statistical software package for Windows, version 12.0 (SPSS Inc, Chicago, Ill). The χ² test procedure tests the hypothesis that observed frequencies do not differ from their expected values. In this study we used the χ² procedure to test the assumption that 50% of the image pairs were diagnosed correctly by the 4 observers. Reliability (interobserver agreement) data were produced in the form of the κ statistic, which takes a value between 0 (no agreement) and 1 (perfect agreement); reliability was therefore assumed to be highly specific at κ>0.8; excellent at κ>0.6; moderate at κ>0.4; and poor at κ≤0.4. Logistic regression analysis was performed on a combination of all morphologic features using the forward-stepwise (Wald) method.

RESULTS

QUANTITATIVE DESCRIPTION OF MORPHOLOGIC CLS MICROSCOPIC FEATURES

General morphologic features such as epidermal and/or dermal involvement and location, shape, and size of the cancer area could be clearly delineated using the composite image procedure provided by the imaging system of the confocal microscope (Figure 1). Regions of interest were further investigated using the field-of-view scan, which is similar to the procedure used for examining histopathologic sections. Individual nuclei were accurately visualized, allowing the distinction to be made between cancerous and normal cells (Figure 2). Furthermore, distribution patterns of individual tumor cells such as peripheral palisading and disorderly patterns within the tumor masses could be recognized (Figure 3). Tumor cell nests appeared well demarcated, often exhibiting peritumoral lacunae (Figure 4).

Within the study set of 20 lesions, 1 superficial, 1 fibrosing, and 18 nodular BCCs were found. In the nodular BCCs, morphologic features like connections between tumor cell formations and the surface epidermis, palisade ar-
rangement of the peripheral cell layer, large aggregates of tumor cells, disintegration of cells in the center of the tumor masses resulting in cyst formation, good or poor demarcation of the tumor from the stroma, and various tumor cell formations could be clearly delineated and recognized. In contrast, the superficial BCC showed buds and irregular proliferations of tumor tissue attached to the undersurface of the epidermis and atrophy of the overlying epidermis. In the fibrosing BCC, a much greater connective tissue participation was found than in the other types of BCC. Normal skin structures, such as epidermis, papillary and reticular dermis, collagen fibers, and adnexal structures including hair follicles, sweat ducts, and sebaceous glands could also be identified and a good comparison made with the morphologic characteristics seen in conventional histopathologic images (Figure 5).

DIAGNOSTIC VALIDATION OF THE METHOD

When 60 confocal image pairs (3 per patient) representing BCC and normal skin each were evaluated by the 4 observers who considered all morphologic features for diagnostic decisions, 2 observers (1 resident and 1 dermatopathologist) correctly classified all image pairs and the remaining 2 observers misclassified only 1 image pair each. These results differ significantly from an expected value of 50% correctly classified image pairs that would occur from pure chance ($P<.001$ for each observer using the $\chi^2$ test).

When the presence or absence of morphologic features was assessed by 2 observers in each image regardless of the diagnosis, tumor cell nuclei and tumor nests were the most useful diagnostic characteristics. Disintegration of tumor cells, palisade arrangement of the peripheral tumor cell layer, and retraction of stroma were less specific and sensitive (Table). Logistic regression analysis of a combination of all features found that tumor nests were highly specific and sensitive. Tumor cell nuclei were also identified as good working diagnostic features. In contrast, disintegration of tumor cells, palisade arrangement of peripheral tumor cell layer, and retraction of stroma were of minor importance. When each feature was measured for its reliability (interobserver agreement) by using the $\kappa$ statistic, most of the diagnostic criteria were highly reliable, indicating good definitions of the morphologic features (Figure 6).

TIME REQUIREMENTS

Preparation of a fresh excision took approximately 1 minute, including cutting a 0.5-mm-thick section and washing with 5% acetic acid for 30 seconds. Fixing the adapter ring on a glass slide and placing the objective lens onto the ring was done in 1 minute. With the composite image function of the system used in this study, 5 to 10 maps including the entire section in most cases were created in approximately 3 minutes. Overall, the total time required to perform a confocal examination of a surgical excision was about 5 minutes.

COMMENT

The present study demonstrates the application of CLS microscopy to diagnostic classification of untreated fresh specimens of BCC in microscopy-guided surgery. Acetic acid–washed sections of tumors and normal skin were imaged using CLS microscopy and evaluated in an observer-blinded manner. In contrast to the procedure followed in Mohs micrographic surgery, vertical sections similar to those used in a conventional histotechnical procedure were prepared. Consequently, morphologic features could be assessed according to the well-known criteria used in conventional histopathologic analysis.

Four independent observers received a standardized 30-minute instructional presentation about diagnostic CLS microscopic features and evaluated 120 confocal images of BCC and normal skin (3 image pairs per patient) imaged with the commercially available Vivascope 1000 confocal microscope. None of the observers in this study had been formally trained in CLS microscopy; none had any previous experience with this method. Furthermore, the analysis was sterile and artificial in that no clinical or histologic diagnosis was taken into account. It is important to note that the morphologic features used for evaluating the test set are easy to learn and use, and this
is reflected in the interobserver agreement and the excellent diagnostic performance of not only the dermatopathologists but also the 2 residents.

Confocal and histopathologic morphologic characteristics seem to correspond well: conventional microscopic features of BCC and normal skin were applied to our confocal image examination. Tumor cell nuclei and tumor nests, as expected, were highly specific, sensitive, and reliable features. Evaluation of disintegration of tumor cells, palisade arrangement of the peripheral tumor cell layer, and retraction of stroma had less diagnostic importance, although their reliability was high when statistically evaluated. Interestingly, logistic regression analysis of the features revealed that evaluating only 1 singular feature (eg, tumor cell nuclei or tumor nests) could be sufficient to reach diagnostic performance similar to that achieved by the observers taking into account all morphologic features for their diagnostic decisions.

Figure 5. Skin features examined under confocal microscopy. Note that normal skin architecture (A) and structures such as collagen fibers (B), hairs (C), sweat ducts (D), and sebaceous glands (E) compare well with the morphologic features found in conventional histopathologic images.
The main advantage of CLS microscopy is the unique opportunity to image thin sections of fresh surgical excisions without processing the tissue for standard histologic analysis. Tissue preparation for confocal examination is simple and time saving. Excisions are cut quickly into thin sections and washed with acetic acid for 30 seconds. When the objective lens is placed onto an adapter ring, which is fixed on the lesion, real-time images can be obtained in seconds in the operating room. This procedure takes approximately 5 minutes.

The time requirement to prepare confocal sections of surgical excisions compared well with that achieved by Rajadhyaksha et al.8 Using a similar procedure, they prepared confocal mosaics within 5 to 6.5 minutes. In contrast, preparation of frozen, hematoxylin-eosin–stained histopathologic specimens requires 22 to 43 minutes for each excision during Mohs micrographic surgery.

The resolution of the confocal microscope is equal to that of conventional microscopes used to view histologic slides, and cellular and architectural details can be examined. Confocal image maps can be rapidly made by the imaging system and provide an overview in large lesions allowing the physician to detect suspected cancerous areas, which can then be evaluated more closely for diagnostic morphologic features. This is similar to the procedure used for histopathologic analysis.

Our study has several limitations. The small number of lesions does not guarantee that the whole range of BCCs is represented. Both residents and dermatopathologists received standardized instruction about specific morphologic features, but difficulties inherent in assessment of large horizontal specimens of mixed tumour types were not assessed. Furthermore, one cannot conclude from the results of 4 independent observers that similar classification results would be achieved by most dermatologists. Finally, in this study each case was represented by preselected images. It is possible that the evaluation of a larger number of images per case taken from various areas of the tumor might not add to the diagnostic accuracy, but might, on the contrary, distract the observers from the correct diagnosis. To evaluate the feasibility of the method further, a large-scale study with more cases and observers and inclusion of the whole set of images obtained in each case would be helpful. The results of the present study, however, provide a set of well-described morphologic criteria with obvious diagnostic impact that should be used in future investigations.

The results of this study demonstrate the possible applications of confocal microscopy in detecting tumor nests in fresh skin biopsy specimens. The speed with which confocal examination of excisions can produce accurate results improves the physician’s ability to manage surgical pathologic analysis and may guide microsurgery of any skin cancer.

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