Quantification of Nucleolar Organizer Regions in Skin Tumors

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Background **: Nucleolar organizer regions (NORs) have recently attracted much attention because of claims that their frequency within nuclei is significantly higher in malignant cells than in normal, reactive, or benign neoplastic cells.**

Objective **: The purpose of this paper is to analyze a method allowing selection of the best morphometric criterion for quantifying AgNOR proteins under conventional observation conditions by light microscopy.**

Method **: We tried to investigate the various parameters including NORs counting in cutaneous tumors using image analysis system.**

Results **: There were significant differences in mean nucleus area per a AgNOR, nucleus area between the benign and potentially malignant group. But the conventional counting of AgNORs is not able to differentiate between the two groups. We could discriminate squamous cell carcinoma from Bowen's disease using parameters of mean ratio of AgNORs area per nucleus area, mean ratio of greatest AgNORs area per nucleus area, coefficient of variation (C V) of nucleus area, and mean area of largest AgNORs. In squamous cell carcinoma and keratoacanthoma, C V of nucleus area has shown a significant difference.**

Conclusion **: Study of AgNORs using image analysis system is a useful tool for the diagnosis of cutaneous tumors. (Ann Dermatol 6:(2) 140-145, 1994)**

Key Words: Skin tumor, Image analysis system

Nucleolar organizer regions (NORs) are loops of DNA that transcribe to ribosomal RNA; they can be easily identified in paraffin section using the silver method[1-3]. Staining of nucleolar organizer regions (NORs) with a silver colloidal method was recently reported to give useful information on the benign or malignant potential of a given tumor[4-9]. However many authors indicated a significant overlapping of NORs counts between benign and malignant proliferation[10,11]. Therefore we tried to investigate the various parameters including NORs counting in cutaneous tumors using image analysis system (AIC., Rosewell, GA)[12-15].

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MATERIAL AND METHOD

Forty-six cutaneous tumors were studied. They comprised : 16 squamous cell carcinoma, 16 seborrheic keratosis, 10 Bowen's disease, 4 keratoacanthoma. These were taken from routine files at Anam Hospital of Korea University. 4um sections of routinely processed, formalin-fixed, paraffin-embedded blocks were cut, dewaxed in xylene, and then hydrated through graded ethanol. The silver colloid solution for staining of NORs was prepared by dissolving gelatin in 1 percent aqueous formic acid at a concentration of 2 percent; this solution was mixed, 1:2 volumes, with 50 percent

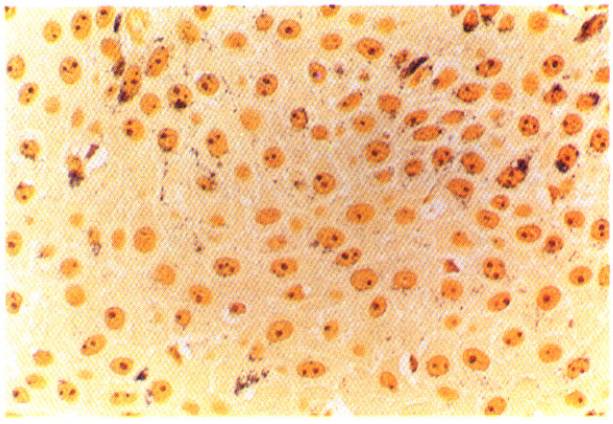

Fig. 1. Same sized-AgNORs are seen in silver staining of seborrheic keratosis(1,000).

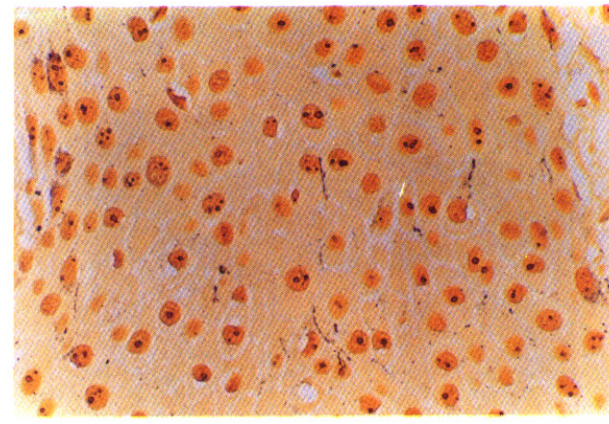

Fig. 2. One or two AgNORs are seen in silver staining of Bowen's disease(1,000).

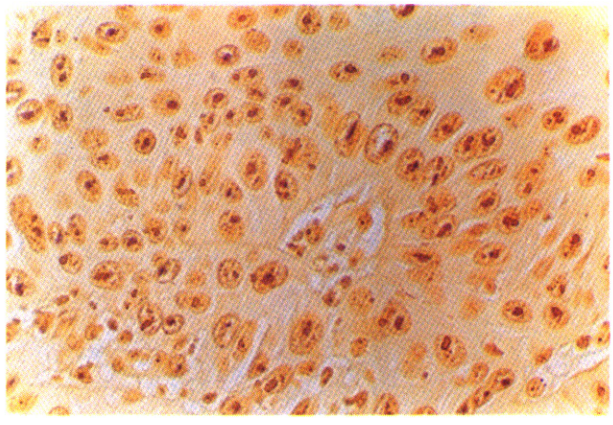

Fig. 3. Many large AgNORs are seen in silver staining of keratoacanthoma(× 1,000).

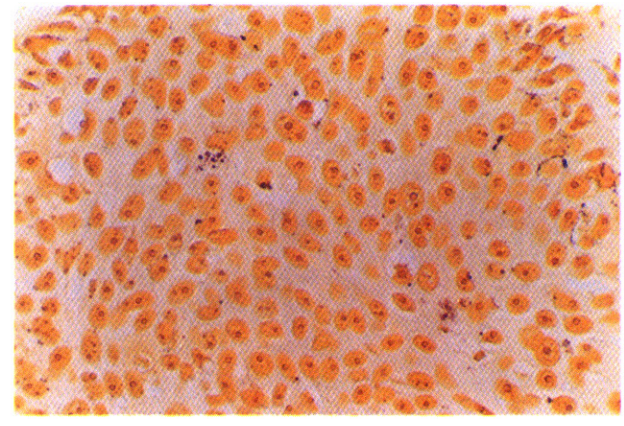

Fig. 4. Silver staining of squamous cell carcinoma(× 1,000).

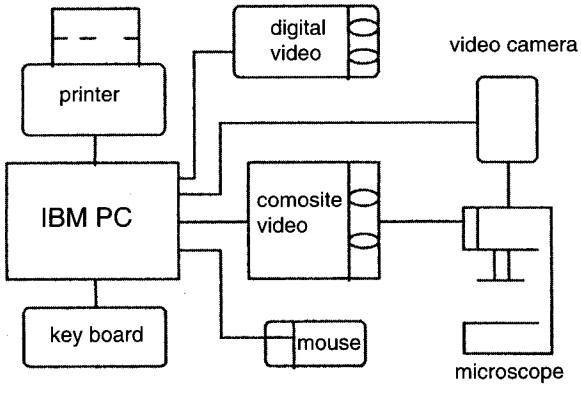

Fig. 5. Schematic diagram of the computerized digital image analysis system.

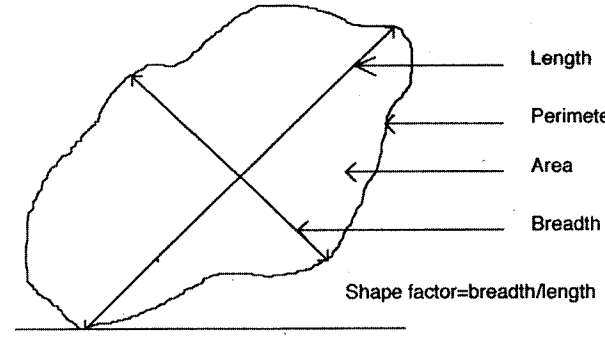

Fig. 6. Diagram of basic parameter.

Table 1. Comparison of the calculated 10 parameters in cutaneous tumors

Parameters	Type		Results	Group
Mean numbers of AgNORs per nucleus	K	A	2.7±1.3	A
	S	C	2.7±1.0	A
	S	K	2.9±1.3	A
	Bowen		2.5±1.0	A
Mean ratio of AgNORs area per nucleus area	K	A	0.11±0.02	A
	S	C	0.11±0.02	A
	S	K	0.08±0.02	B
	Bowen		0.07±0.01	B
C V of mean ratio of AgNORs area per nucleus area	K	A	0.34±0.04	A
	S	C	0.35±0.07	A
	S	K	0.34±0.05	A
	Bowen		0.35 0.05	A
Mean ratio of greatest AgNORs are per nucleus area	K	A	0.10±0.02	A
	S	C	0.09±0.02	A
	S	K	0.08±0.02	B
	Bowen		0.06 0.01	B
Mean nucleus area per a AgNOR	K	A	20.16±2.82	A
	S	C	17.82±4.14	A
	S	K	11.82 2.74	B
	Bowen		17.83 5.34	A

* $p<0.01$ except mean numbers of AgNORS per nucleus and C V of mean ratio of AgNORs area per nucleus area
* compared by ANOVA and Duncan's multiple range test
* means with the same letter are not significantly diferent
* K A: keratoacanthoma S C: squamous cell carcinoma S K: seborrheic keratosis Bowen: Bowen's disease

aqueous silver nitrate to obtain the final working solution. This was dropped onto the sections and left for 45 minutes under safelight conditions at room temperature, after which the sections were washed with deionized water. The sections were taken to xylene for dehydration and mounted in a synthetic medium[6].

The AgNORs were seen as dots within the nuclei of the cells (Fig. 1.-4), and in each case 100 cells were examined using an x 100 oil immersion lens. Each slide was analyzed with image analysis system (Fig. 5, AIC Inc., Roswell, GA). The five basic parameters automatically analyzed and stored in a data file for each AgNOR and nucleus were length, breadth, shape factor, perimeter, and area (Fig. 6). On the basis of these measured values, 10 morphologic parameters such as mean numbers of AgNORs per nucleus, mean ratio and CV of AgNORs area per nucleus area, mean ratio of greatest AgNORs area per nucleus area, AgNORs area, CV of AgNORs area, nucleus area, CV of nucleus area, mean area of largest AgNORs, mean nucleus area per a AgNOR were computed. Statistical evaluation was performed with ANOVA.

RESULTS

There were significant differences in mean nucleus area per a AgNOR, nucleus area between benign(seborrheic keratosis) and potentially malignant group(squamous cell carcinoma, keratoacanthoma, Bowen's disease). But the conventional counting of AgNORs(mean numbers of AgNORs per nucleus) is not able to differentiate between the two groups(Table 1,2). We could discriminate squamous cell carcinoma from Bowen's disease using

Table 2. Comparison of the calculated 10 parameters in cutaneous tumors

Parameters	Type		Results	Group
AgNORs area	K	A	4.61±0.99	A
	S	C	4.26±1.48	A
	S	K	2.62±0.92	B
	Bowen		2.55±0.98	B
C V of AgNORs area	K	A	0.51±0.15	A
	S	C	0.48±0.13	A
	S	K	0.41±0.09	A
	Bowen		0.45±0.04	A
Nucleus area	K	A	54.19 7.25	A
	S	C	48.12 11.18	A
	S	K	34.29 7.96	B
	Bowen		44.56 13.34	A
C V of nucleus area	K	A	0.299 0.06	A
	S	C	0.238 0.06	B
	S	K	0.229 0.05	B
	Bowen		0.252 0.05	A
Mean area of largest AgNORs	K	A	5.35 1.45	A
	S	C	4.71 1.61	A
	S	K	2.76 1.08	B
	Bowen		2.77 0.96	B

* $p<0.01$ except C V of AgNORs area
* compared by ANOVA and Duncan's multiple range test
* means with the same letter are not significantly different
* K A: keratoacanthoma S C: squamous cell carcinoma
  S K: seborrheic keratosis Bowen: Bowen's disease

parameters of mean ratio of AgNORs area per nucleus area, mean ratio of greatest AgNORs area per nucleus area, coefficient of variation(C V) of nucleus area, and mean area of largest AgNORs (Table 1, 2). In squamous cell carcinoma and keratoacanthoma C V of nucleus area has shown a significant difference(Table 2).

DISCUSSION

Nucleolar organizer regions(NORs) have recently attracted much attention because of claims that their frequency within nuclei is significantly higher in malignant cells than in normal, reactive, or benign neoplastic cells 4-9. Because NORs can now be demonstrated relatively easily in routinely processed histological sections, the technuque is obviously of potential value in diagnostic histopathology 6. NORs are chromosomal segments in which ribosomal RNA(rRNA) is encoded and located on each of the short arms of the acrocentric chromosomes 13, 14, 15, 21, and 22[16].

The silver-staining technique identifies neither rDNA nor rRNA but the acidic proteins associated with these sites of rRNA transcription; these proteins are designated B23, C23, AgNOR protein and RNA polymerase I. The structures thus demonstrated are termed AgNORs'[17].

The abundance and intensity of AgNORs are an indication not only of their absolute number and dispersion but also their transcriptional activity[18]. A quantifiable apparent increase in the mean AgNOR count of a cell population in tissue sections could result if either: (a) cell proliferation is so active that nucleolar dissociation is present in many cells and the AgNORs are therefore dispersed through the nucleus; (b) there is a defect of nucleolar association resulting in AgNOR dispersion; (c) cell

ploidy increase, resulting in a real increase of AgNOR-bearing chromosomes; or (d) transcriptional activity increase resulting in prominence of otherwise inconspicuous AgNORs [18].

AgNOR counts are reported to assist in the distinction between high- and low- grade lymphoma 5, benign melanocytic lesions from malignant melanoma[19], and reactive mesothelial proliferation from mesothelioma[18]. But there is in many instances a variable degree of overlap between benign and malignant or high- and low- grade lesions, prohibiting use of the AgNOR technique as an absolute sole criterion.

Therefore we tried to investigate the (same paragraph) various parameters including NORs counting in cutaneous tumors using image analysis system[14]. Until now, very few studies have been performed with image analyzers because they are costly and not devoted specifically to AgNOR dot counting. Quantification of AgNOR dots is still in its early stages. Several authors use their "own" parameter a representative of NOR content, but very few comparative results based on different parameters have been reported. Our aim is to avoid any a prior parameter and to try to define as objectively as possible which parameter is important or useful in a given situation. For this reason, we computed several parameters for the same cell. Our work demonstrated that reproducible quantification of AgNOR dots may be performed routinely by using image analysis system. Numerous parameters may be used, and we focused our attention on methods allowing the standardization of quantification and rapid and efficient statistical analysis of the results. The potential usefulness of the image analysis system was demonstrated for cutaneous tumors in which AgNOR dot counting was not useful as a discriminate parameter. In conclusion, the study of AgNORs using image analysis system is a useful tool for the diagnosis of cutaneous tumors.

REFERENCES

1. Hubert J, Bourgeois CA, Bouteille M: Ultrastructural localization of AgNOR stained proteins in the nucleolus during the cell cycle and in other nucleolar structures. Chromosoma 1980;79:349-362
2. Busch H, Daskai Y, Gyorkey F, et al: Silver staining of nucleolar granules in tumor cells. Cancer Res 1979;39:857-863
3. Wachtler F, Hopman AHN, Wiegant J, et al: On the position of Nucleolar organizer regions in interhpase nuclei. Exp Cell Res 1986;167:227-240
4. Crocker J, Skilbeck N: Nucleolar organizer region associated proteins in cutaneous melanotic lesions:a quantitative study. J Clin Pathol 1987;40:885-889
5. Crocker J: Proliferation indices in malignant lymphomas. Clin Exp Immunol 1989;77:299-308
6. Crocker J, Nar P: Nucleolar organizer regions in lymphomas. J Pathol 1987;151:111-118
7. Crocker J, Mcgovern J: Nucleolar organizer regions in normal, cirrhotic, and carcinomatous livers. J Clin Pathol 1988;41:1044-1048
8. Morgan DW, Crocker J, Watts A, et al: Salivary gland tumours studied by means of the AgNOR technoque. Histopathology 1988;13:553-559
9. Crocker J, Egan MJ: Correlation between NOR sizes and numbers in Non-Hodgkin's lymphomas. J Pathol 1988;156:233-239
10. Deschenes J, Weidner N: Nucleolar organizer regions (NOR) in hyperplastic and neoplastic prostate disease. Am J Surg Pathol 1990;14:1148-1155
11. Jun C, Han Kyeum K: Silver-binding Nucleolar Organizer Regions(AgNORs) in benign and malignant prostatic lesions. New Med J 1992;35:35-41
12. Kee Huem N,Chil Hwan O,Soo Nam K : A study of the Quantitative and Morphological change of elastic fiber due to an aging process by using a computerized digital image analysis system. Kor U M J 1991;28:867-878
13. Martin H, Hufnagl P, Beil M, et al: Nucleolar organizer region-associated proteins in cancer cells. Anal Quant Cytol Histol 1992;14:312-319
14. Ploton D, Viesseaux B, Canellas JC, et al: Semiautomatic quantification of silver-stained nucleolar organizer regions in tissue sections and cellular smears. Anal Quant Cytol Histol 1992;14:14-23
15. Lesty C, Chleq C, Contesso G, et al: Nucleoli and AgNOR proteins in 32 cases of primary breast carcinoma. Anal Quant Cytol Histol 1992;14:175-186
16. Troster H, Spring H, Meissner B, et al: Structural organization of an active, chromosomal nucleolar organizer region identified by light microscopy, and subsequent TEM and STEM electron microscopy. Chromosoma 1985;91:151-163
17. Ploton D, Menager M, Adnet J: Simultaneous high resolution loca lization of AgNOR proteins and nucleoproteins in interphasec and mitotic nuclei. J Histochem 1984;16:897-906

18. Wiley J: Nucleolar organizer regions as diagnostic discriminants for malignancy. J Pathol 1988; 155:95-96
19. Gambini C, Casazza C, Borgiani L, et al: Counting the nucleolar organizer region-associated proteins is a prognostic clue of malignant melanoma. Arch Dermatol 1992;128:487-490