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► To cite this version:

Audrey Benyamine, Daniel Bertin, Xavier Heim, Brigitte Granel, Nathalie Bardin. Should we look for anti-RNA polymerase III antibodies in systemic sclerosis patients with anti-centromere or anti-topoisomerase I antibodies?. *European Journal of Internal Medicine*, 2017, 44, pp.e42-e44. 10.1016/j.ejim.2017.07.033 . hal-01792225

HAL Id: hal-01792225

<https://amu.hal.science/hal-01792225>

Submitted on 18 May 2018

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3 **Should we look for anti-RNA polymerase III antibodies in Systemic Sclerosis patients**4 **with anti-centromere or anti-topoisomerase I antibodies?**5 *Running title: anti-RNA polymerase III in Systemic Sclerosis*

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22 The authors declare no conflict of interest.

23 **Keywords:** Systemic Sclerosis; Anti-RNA Polymerase III antibodies; Anti-centromere

24 Antibodies; Anti-topoisomerase I antibodies; Immunofluorescence pattern

25 Systemic sclerosis (SSc) is a chronic autoimmune disease characterised by skin and internal
26 organs fibrosis, vascular damage and positive antinuclear autoantibodies (ANAs). The
27 classical antigenic specificities of ANAs are anti-centromere (ACA), anti-topoisomerase I
28 antibodies (anti-topo I) and the more recently described anti-RNA polymerase III antibodies
29 (anti-RNAPIII).

30 Anti-RNAPIII, firstly described in SSc in 1993[1], are currently admitted as specific SSc-
31 related autoantibodies and have been incorporated into the 2013 ACR/EULAR classification
32 criteria for the disease [2]. The prevalence of anti-RNAPIII partially depends on the
33 geographic origin of patients [3]. In France, the prevalence is low and ranges from 3 to 9%
34 whereas it can reach 14% in North America and 41% in South America [3,4]. The ANA
35 pattern associated with anti-RNAPIII was described as a fine-speckled nuclear stain with
36 additional occasional bright dots, with or without concurrent punctate nucleolar staining but
37 no typical pattern was proposed [5]. Anti-RNAPIII are mainly associated with a diffuse
38 cutaneous subtype, scleroderma renal crisis and a risk of cancer in close temporal relationship
39 to SSc onset [3,4,6,7]. As the specific method for their identification was the
40 radioimmunoprecipitation assay, a cumbersome method not suitable for routine practice, these
41 autoantibodies were not routinely looked for. More recently immunoenzymatic methods have
42 been developed, but, the detection strategy was not clearly established for routine practice [5].
43 Although the positivity of ACA and anti-topo I is considered to be exclusive, the co-positivity
44 of anti-RNAPIII with these SSc-specific auto-antibodies remains to be clarified. In order to
45 design the best strategy for anti-RNAPIII detection, we aimed to 1/ evaluate the co-positivity
46 of anti-RNAPIII in SSc patients positive for ACA or anti-topo I and to 2/ analyse
47 immunofluorescence patterns and clinical characteristics of anti-RNAPIII positive patients.

48

49 Firstly, 76 sera from SSc patients (9 men, 67 women) from Marseilles (South of France)
50 positive for either ACA or anti-topo I antibodies were tested for the presence of anti-
51 RNAPIII. All patients fulfilled the 2013 ACR/EULAR criteria and were further classified as
52 having diffuse or limited cutaneous SSc [2]. All the sera were collected from 2012 to 2016
53 and were issued from a Biobank (DC 2012-1704) with respect of ethical directives.
54 Antinuclear antibodies (ANAs) were detected by indirect immunofluorescence on HEp-2 cells
55 (Bio-Rad Laboratories, Hercules, CA) at a screening dilution of 1:160. ACA, anti-topo I and
56 anti-RNAPIII were detected by commercially kits (EliA Thermo Fisher). The cutoff value for
57 anti-RNAPIII positivity was 10 Arbitrary Unit/ml (AU).

58 Secondly, the ANAs immunofluorescence patterns and clinical data of 8 SSc patients positive
59 for anti-RNAPIII were collected from 2012 to 2016 and compared to anti-RNAPIII negative
60 SSc patients (<10 AU/ml). Results were expressed as median +/- interquartile range or as
61 frequencies (fq). Medians were compared using Mann Whitney U Test. Frequencies were
62 compared using Chi 2 Test.

63 Among the 76 selected sera of SSc patients, 33 patients (43%) were positive for ACA and 43
64 (57%) for anti-topo I antibodies. Immunofluorescence nuclear patterns were: centromeric
65 ($n=32$), nucleolar homogeneous ($n=41$), speckled-centromeric ($n=1$), speckled-homogeneous
66 ($n=1$) and mixed speckled-nucleolar-centromeric ($n=1$). Anti-RNAPIII were investigated in
67 these SSc patients: only one ACA-positive serum (1.3%) was found also positive for anti-
68 RNAPIII with a titer of 192 AU/ml. This serum corresponded to the mixed speckled-
69 nucleolar-centromeric immunofluorescence nuclear pattern (**Figure 1**). No other specificities
70 associated with a speckled pattern (anti-Ro/SSA anti-La/SSB, anti-Sm, anti-RNP) were
71 detected. The patient, a 47-year-old female, had a diffuse cutaneous subset, with digital
72 ulcers, joint contractures, a reduced diffuse lung capacity for carbon monoxide and
73 oesophageal reflux disorder. Her medical history was remarkable for an ovarian

74 adenocarcinoma that was diagnosed 6 years before SSc, and considered in remission after
75 surgery and radio-chemotherapy.

76 Then, we retrospectively analysed immunofluorescence pattern and clinical characteristics of
77 8 SSc patients found positive for anti-RNAPIII in our laboratory. The various
78 immunofluorescence aspects were nuclear speckled ($n=5$), nucleolar ($n=1$), nucleolar-
79 speckled ($n=1$) (**Figure 2**) and mixed speckled-nucleolar-centromeric ($n=1$) (**Figure 1**). The
80 median titer of anti-RNAPIII was 54 [18-298] AU/ml.

81 **Table 1** illustrates the comparison between anti-RNAPIII positive and negative patients. Sex
82 ratio did not differ between the two groups. A trend to a higher frequency of the diffuse
83 cutaneous form was observed in anti-RNAPIII positive SSc patients. The two patients with
84 *sine scleroderma* belonged to the group of negative anti-RNAPIII. Scleroderma renal crisis
85 was solely documented in patients with positive anti-RNAPIII. Other variables were not
86 significantly associated with anti-RNAPIII positivity.

87 The present study extends the previously published data performed in South of France [4]
88 about the anti-RNAPIII screening strategy. This study highlights that anti-RNAPIII are rarely
89 encountered in SSc patients already positive for ACA or anti-topo I antibodies. The case of
90 the patient with coexisting ACA and anti-RNAPIII was interesting regarding two aspects.
91 First, the clinical feature was closer to the one described in patients with anti-RNAPIII with a
92 diffuse cutaneous form and a concomitant cancer [8]. Second, the observed ANAs
93 immunofluorescence pattern was highly remarkable due to the peculiar aspect of
94 immunofluorescence. Therefore, in ACA or anti-topo I positive sera, the search for anti-
95 RNAPIII can be recommended faced to a mixed fluorescence pattern. Conversely, in case of
96 typical fluorescence aspects related to ACA or anti-topo I positivity, the systematic search for
97 anti-RNAPIII is not mandatory. As observed herein, different immunofluorescence pattern

98 can be associated with anti-RNAPIII [9]. Therefore the search for this auto-antibody should
99 not be restrained to a nucleolar immunofluorescence pattern of ANAs [10].

100 Regarding the phenotype of anti-RNAPIII positive patients, a higher frequency of
101 scleroderma renal crisis and diffuse cutaneous form was observed. The low number of
102 patients and our geographic location might be a limiting factor to evidence any association
103 with other anti-RNAPIII features such as the frequency of cancer [8].

104 Although SSc-related autoantibodies are exclusive markers, co-positivity can exist in rare
105 cases. The immunofluorescence reading step appears crucial to focus the screening. In order
106 to improve the benefit cost ratio, anti-RNAPIII should be searched with respect to ANAs
107 fluorescence pattern and clinical characteristics of the patients.

108

109 **ACKNOWLEDGEMENT**

110 This research did not receive any specific grant from funding agencies in the public,
111 commercial, or not-for-profit sectors.

112 We thank internal medicine physicians for their help in collecting the clinical data (M.Ebbo,
113 N.Schleinitz, G.Kaplanski, C.Gomez).

114

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153

154

155 **Table 1: Characteristics of SSc patients with respect to positivity of the anti-RNA**
 156 **Polymerase III (anti-RNAPIII) autoantibodies.**

Characteristics	Anti-RNAPIII + (n=8)	Anti-RNAPIII- (n=75)	p-value
Sex Ratio (F/M)	6/2	66/9	0.30
Age (years, median) [IQR]	54 [39.5-71.3]	62.4 [49.9-70.6]	0.47
Age at disease onset (years, median) [IQR]	52 [30.5-71.8]	53 [43.8-61.3]	0.77
Age at the apparition of Raynaud phenomenon (years, median) [IQR]	48 [30-70.5]	50 [38-56]	0.97
Diffuse SSc/Limited SSc	5/3	23/50	0.08
Sine Scleroderma SSc	0/8	2/75	0.64
Pulmonary fibrosis (Fq)	2/8	25/70	0.55
FVC (% , median) [IQR]	87.6 [70.2-108.3]	79.5 [68.8-100.5]	0.49
DLCO (% , median) [IQR]	57 [41-65]	51.3 [41.4-63.7]	0.77
DLCO/VA (% , median) [IQR]	67 [48-76]	63.65 [52-73]	0.68
Pulmonary Arterial Hypertension (Fq)	2/8	20/72	0.87
Esophagus Reflux Disorder (Fq)	3/8	24/65	0.97
Intestinal Motility Disorder (Fq)	1/8	26/73	0.26
Medsger Severity Scale (Fq)	3 [3-4]	3 [2-3.37]	0.27
Scleroderma Renal Crisis (Fq)	2/8	0/75	<10 ⁻⁴
Cancer occurrence	1/8	9/75	0.96
Anti-Topo 1 antibody (Fq)	0/8	43/75	-
Anti-centromere antibody (Fq)	1/8	32/75	-

157 Results are expressed as median +/- interquartile range or as frequencies (fq). Medians were
 158 compared using Mann Withney U Test. Frequencies were compared using Chi 2 Test.

159 **Legends of the figures**

160

161 **Figure 1:** The mixed fluorescence pattern (speckled-nucleolar-centromeric) of the ACA and

162 Anti-RNAPIII positive serum.

163 **A:** x 400 magnification.

164 **B:** Typical features of the centromeric pattern are highlighted with characteristic dots at the

165 interphase mitotic stage (left arrow) and a “block” of condensed dots at the metaphase stage

166 (right arrow).

167

168 **Figure 2:** Various immunofluorescence patterns obtained from sera positive for anti-RNAPIII

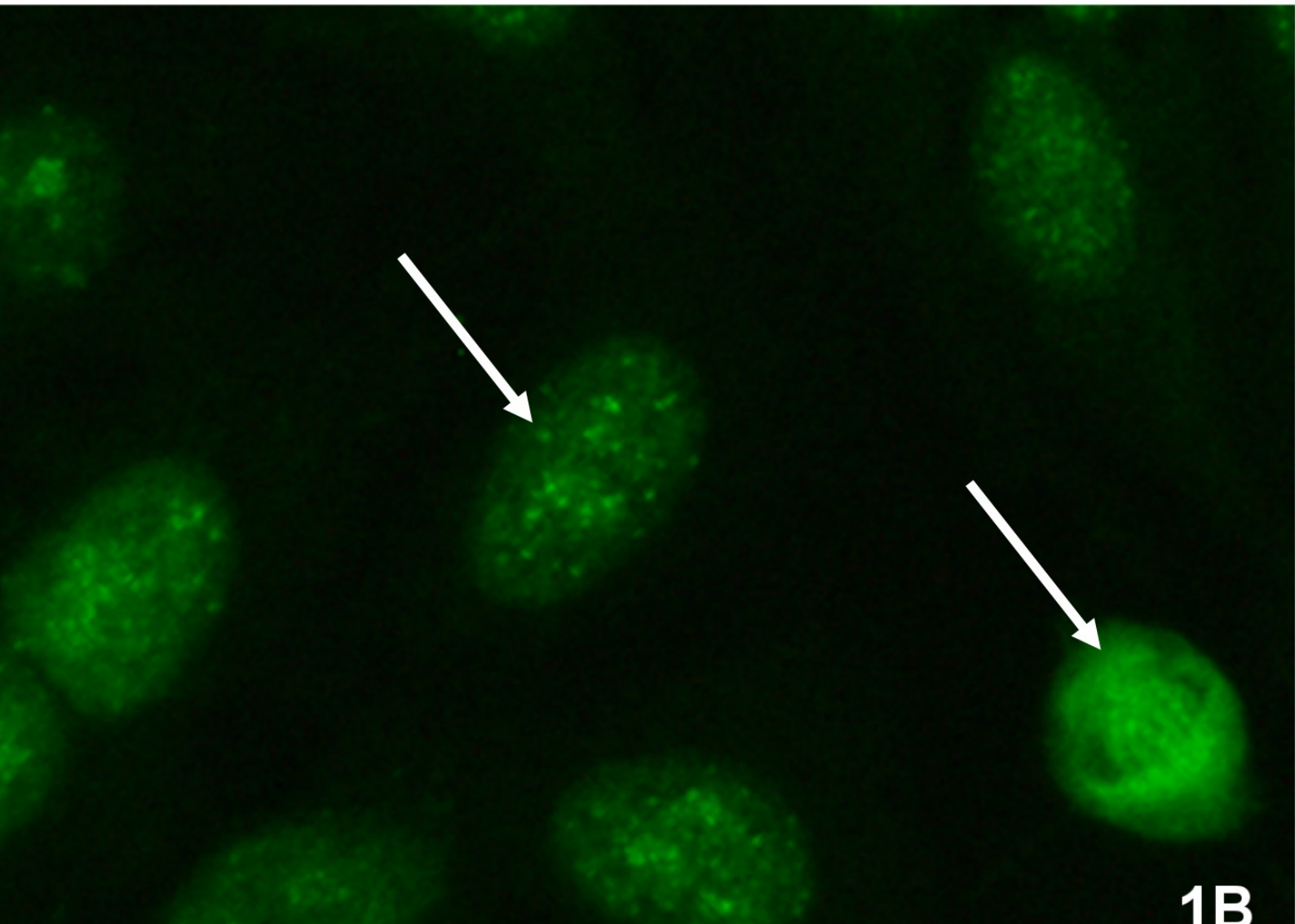
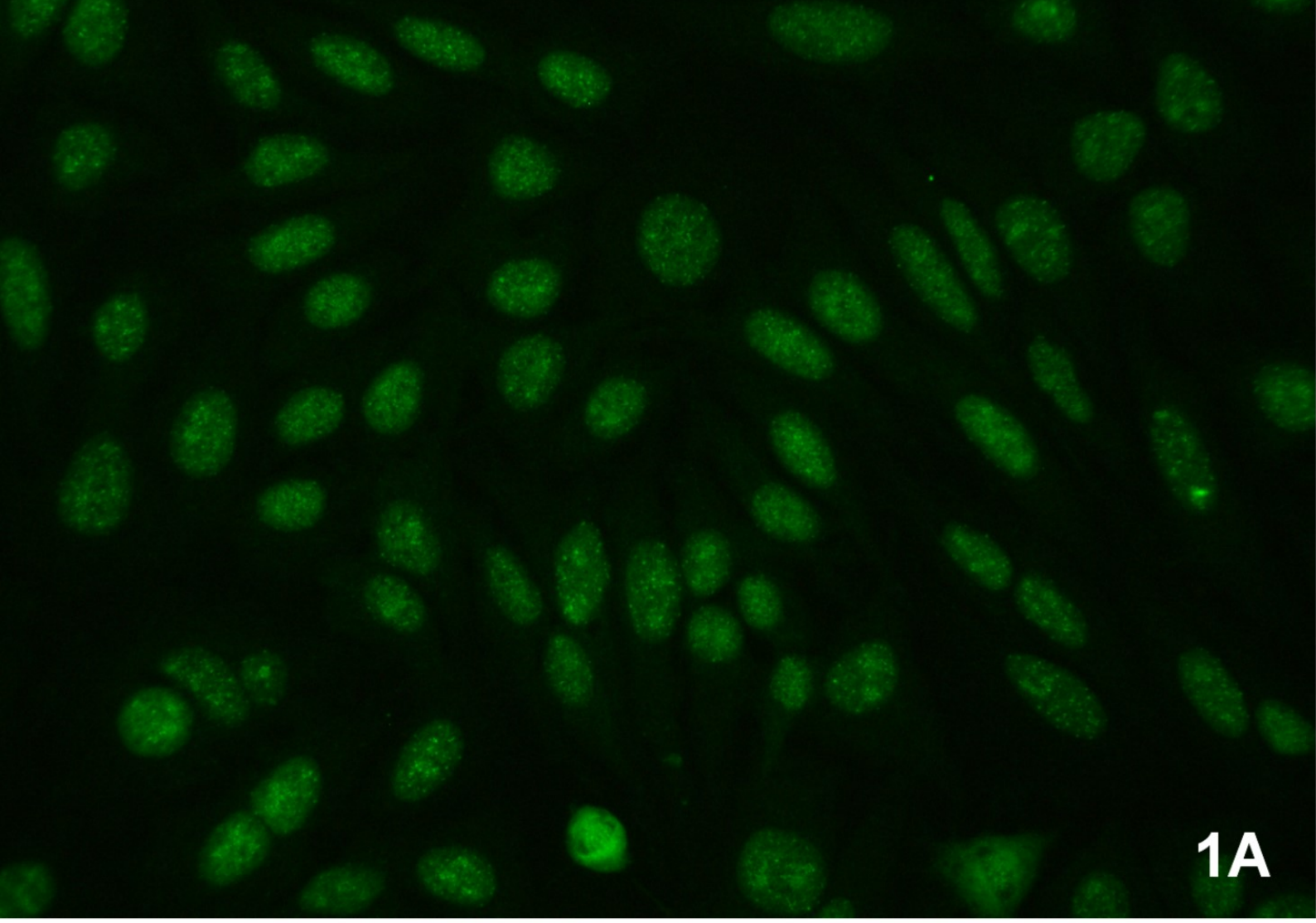
169 (x 400 magnification).

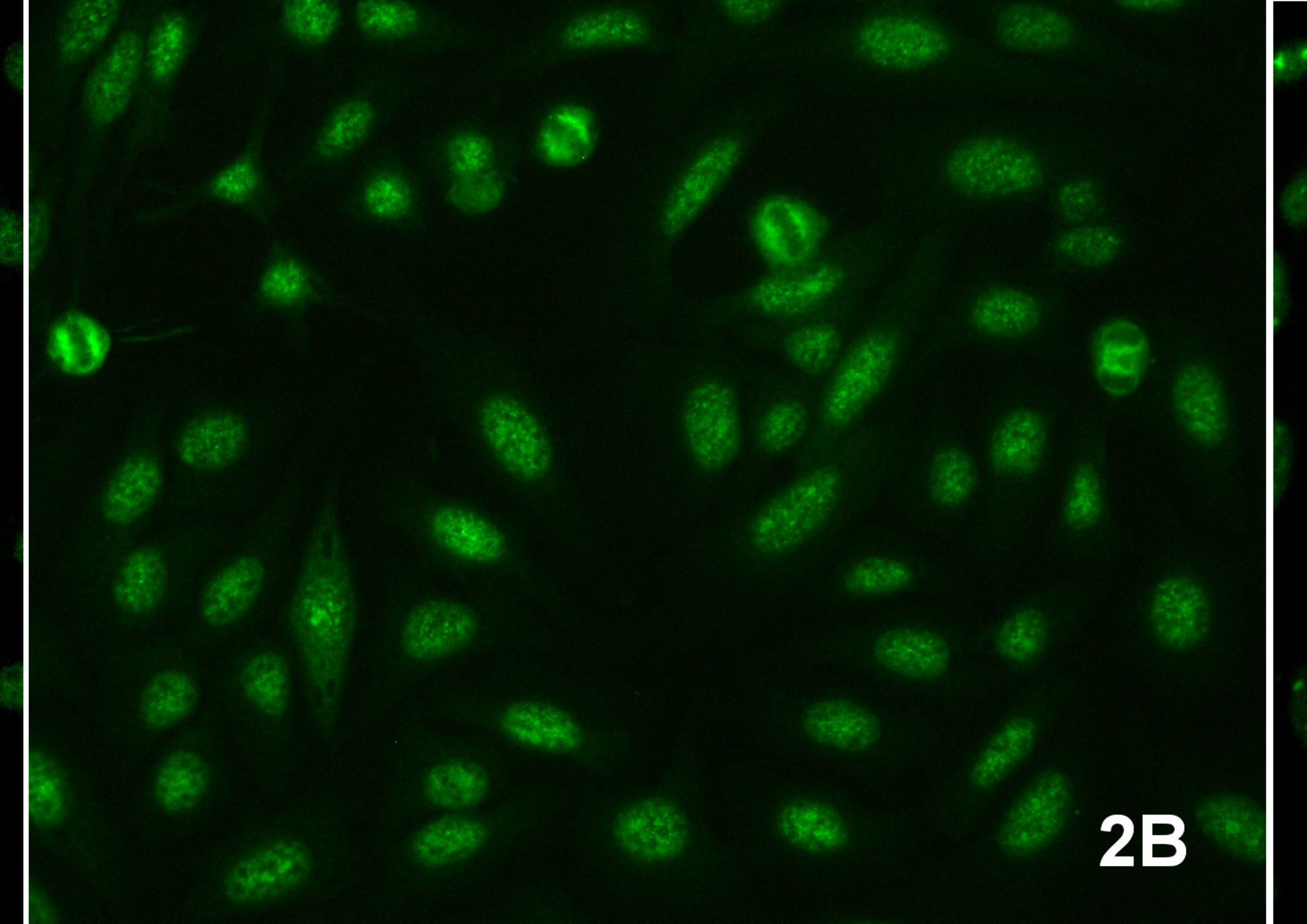
170 **A:** nuclear-speckled pattern

171 **B:** nucleolar-speckled pattern

172 **C:** nucleolar pattern

173





2B