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Original Article

The propensity for tumorigenesis in human induced pluripotent stem cells is related with genomic instability

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Abstract

The discovery of induced pluripotent stem cells (iPSCs) is a promising advancement in the field of regenerative medicine. Previous studies have indicated that the teratoma-forming propensity of iPSCs is variable; however, the relationship between tumorigenic potential and genomic instability in human iPSCs (HiPSCs) remains to be fully elucidated. Here, we evaluated the malignant potential of HiPSCs by using both colony formation assays and tumorigenicity tests. We demonstrated that HiPSCs formed tumorigenic colonies when grown in cancer cell culture medium and produced malignancies in immunodeficient mice. Furthermore, we analyzed genomic instability in HiPSCs using whole-genome copy number variation analysis and determined that the extent of genomic instability was related with both the cells' propensity to form colonies and their potential for tumorigenesis. These findings indicate a risk for potential malignancy of HiPSCs derived from genomic instability and suggest that quality control tests, including comprehensive tumorigenicity assays and genomic integrity validation, should be rigorously executed before the clinical application of HiPSCs. In addition, HiPSCs should be generated through the use of combined factors or other approaches that decrease the likelihood of genomic instability.

Key words Autophagy, fusion oncoprotein, acute myeloid leukemia

The reprogramming of somatic cells by defined factors that produce mouse or human induced pluripotent stem cells (iPSCs) has been well documented[1-5]. This property is of great importance to the field of regenerative medicine because it could potentially provide an unlimited source of individual-specific tissue replacement therapy as well as a new resource for the study of genetic diseases; however, there are safety concerns, especially with regard to their potential tumorigenicity. Indeed, this is the main obstacle delaying the clinical application of human iPSCs (HiPSCs). Previous studies have indicated that the tumorigenic potential of iPSCs is related with expression of the *C-MYC* oncogene[6], the origin of the somatic tissues from which the iPSCs are induced[7,8], and the status of the cells' p53 genotype[9]; however, the complete molecular mechanism underlying tumor potential remains to be elucidated. We performed multiple assays, including *in vitro* colony formation assays and *in vivo* tumorigenicity tests in severe combined immunodeficient (SCID) mice, to estimate the propensity for tumorigenesis in our previously established HiPSC lines[10].

Several reports have illustrated that HiPSCs typically harbor normal karyotypes[3,4,10]. However, in mouse iPS cells, knocking out p53 initiated chromosomal aberrations[11], which is indicative of genomic instability. Signs of genomic instability, including aneuploidy, chromosomal aberrations, and DNA amplification/deletion, have been documented as being hallmarks of cancer[12]. A recent

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global gene expression meta-analysis has demonstrated that chromosomal aberrations exist in HiPSCs[13]. Using a high-resolution, single-nucleotide polymorphism (SNP) analysis, dynamic copy number variation (CNV) changes were detected in human embryonic stem cells (HESCs) and HiPSCs, both during reprogramming and over time through the course of routine cell culture[14,15].

Although we have demonstrated that genomic instability induced common cancer cells to become stem-like cancer cells[16], it remains unclear if there is an intrinsic association between the genomic instability of HiPSCs and their tumorigenicity. Based on the observation that culture-adapted HESCs may form teratocarcinomas, a relationship between chromosomal aberrations and the tumorigenicity of HESCs/HiPSCs has been suggested[17,18]. We have previously established four HiPSC lines (CMC, hNF1-4, Tibia, and UMC) by transfection with four Yamanaka's factors (*OCT4*, *SOX2*, *C-MYC*, and *KLF4*)[1,2]. We validated their pluripotency by alkaline phosphatase (AP) staining, immunostaining for embryonic stem cell markers, bisulfate sequencing for the DNA methylation profile at the *OCT4* and *NANOG* promoters, and teratoma formation in SCID mice[10]. In this study, we used whole-genome CNV analysis to provide direct evidence that the propensity for tumorigenesis in HiPSCs relates with genomic instability.

Materials and Methods

Cell culture

Four HiPSC lines, designated CMC, hNF1-4, UMC, and Tibia, were generated and stored in our laboratory[10]. The cell culturing protocols have been previously described[19]. Briefly, HiPSCs were grown at 37℃ and 5% CO_2 in mTeSR®1 medium (StemCell Technologies) with BD Matrigel™ hESC-qualified Matrix (BD Biosciences) as a substrate.

Colony formation assay

HiPSCs were trypsinized, plated at a density of 1 × 10^6 cells per dish and cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 2 weeks. After the cell clones had expanded to >50 cells, the cells were washed twice with PBS, fixed in methanol for 5 min, and dyed with crystal violet for 10 min at room temperature.

Teratoma formation assay

SCID mice were purchased from the animal institute of the Chinese Academy of Medical Science and maintained in microisolator cages. All experiments were approved by the animal care committee at Sun Yat-sen University. HiPSCs were counted, mixed with 50% Matrigel (BD Biosciences), and subcutaneously transplanted into the flank of 5- to 6-week-old SCID mice. Mice were euthanized 10 weeks after transplantation and assessed for teratoma formation. A portion of the tumor tissue was collected, fixed in 10% formaldehyde, and embedded in paraffin for hematoxylin and eosin (HE) staining to assess tumor pathology. HE staining was performed according to the standard protocol.

Immunohistochemical staining for OCT4

Paraffin-embedded teratoma tissue slides were deparaffinized, rehydrated, and processed with antigen retrieval by boiling the slides in a sodium citrate buffer (10 mmol/L, pH 6.0). The slides were immersed in 3% H_2O_2 for 10 min and washed three times with PBS. The tissue slides were blocked with goat serum for 20 min. The primary antibody, anti-OCT4 (Cell Signaling Technology) diluted in primary antibody dilution buffer (Dako) at 1:100, was then added, and tissue slides were incubated at 4℃ overnight in a humidified container. After washing three times with PBS, the tissue slides were treated with a non-biotin horseradish peroxidase detection system (Dako) and washed three times with PBS. Subsequently, the tissue slides were stained with 3,3-diaminobenzidine (DAB) and counterstained with hematoxylin.

Copy number variation analysis

DNA was isolated from HiPSCs and their parental cells and then hybridized on the Cytogenetics Whole-Genome 2.7M Array in accordance with the manufacturer's protocol (Affymetrix). The hybridization data were analyzed using Chromosome Analysis Suite (ChAS) software, and the CNV loci results were calculated using the Affymetrix reference data as the control. The CNV experimental procedures and data analysis were performed by CapitalBio Corp. in Beijing, China.

Results

Tumorigenic colony formation of HiPSCs

To comprehensively explore the *in vitro* and *in vivo* tumorigenicity of HiPSCs, we first tested the tumorigenic colony-forming abilities of our HiPSC lines. As expected, most HiPSCs did not survive in DMEM supplemented

with 10% FBS, a culture medium often used for the cultivation of cancer cells; cell colonies were only observed for CMC cells after two weeks of culturing (Figure 1A).

To test for tumorigenicity, we injected 5×10^6 HiPSCs into the flank of SCID mice. Palpable tumors, which were proven to be teratocarcinomas by HE staining, emerged 5 weeks after inoculation (Figure 1B).

To determine the differentiation status of the tumors, we performed immunohistochemical staining for OCT4, a marker of undifferentiation in pluripotent cells. OCT4 was highly expressed in tumor cells (Figure 1C), indicating the malignant capacity of CMC-derived cells. Therefore, the CMC-derived HiPSC line could be transformed into teratocarcinoma using cancer cell culture conditions.

Tumorigenic capability of HiPSCs in SCID mice

To further evaluate the capacity of HiPSCs to form teratoma *in vivo*, we subcutaneously transplanted HiPSCs into the flank of SCID mice. Ten weeks after 1×10^6 cells were transplanted, only CMC and hNF1-4 cells produced teratomas; when the cell number was increased to 3×10^6, CMC cells formed tumors in 3 of 4 mice, whereas hNF1-4, Tibia, and UMC cells formed tumors in only 1 mouse, respectively (Table 1).

More importantly, HE staining demonstrated that the extent of differentiation in these teratomas varied. Indeed, CMC cells gave rise to only undifferentiated, cartilage-like tissue (Figure 2A). On the other hand, UMC, Tibia, and hNF1-4 cells, with weak teratoma-forming capability, produced teratomas with well-differentiated tissues and cartilage (Figures 2B–D).

Immunohistochemical staining for OCT4 revealed a relationsip between OCT4 expression and differentiation status. OCT4 was positive in CMC-derived teratomas (Figure 2E), but negative in teratomas derived from UMC, Tibia, and hNF1-4 (Figures 2F–H). Therefore, in addition to forming tumorigenic colonies *in vitro*, HiPSCs may possess the potential to develop into malignancies *in vivo* under certain conditions.

CNV detection in HiPSCs and their parental cells

We previously reported that the HiPSC lines possess a normal karyotype[10]. Therefore, we used whole-genome CNV analysis, which is more sensitive in detecting genomic abnormalities than karyotyping, to

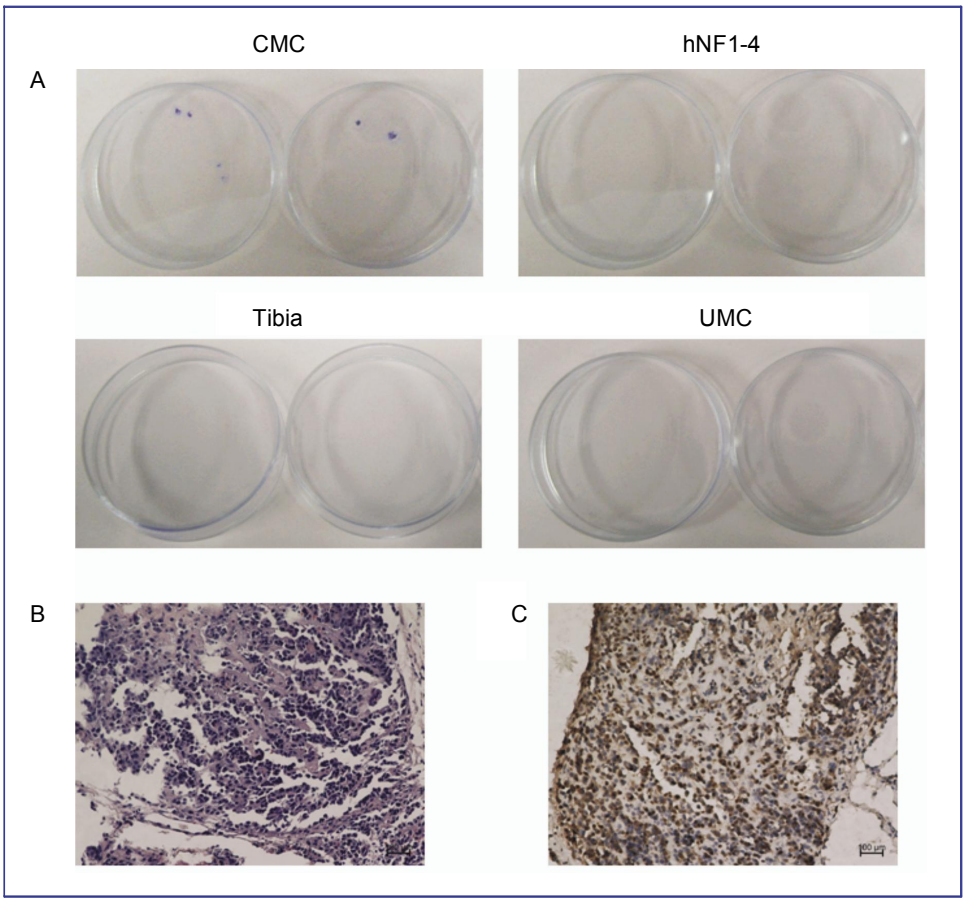

Figure 1 **Human induced pluripotent stem cells (HiPSCs) form tumorigenic colonies in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).** A, representative pictures of the colony formation assays with CMC, hNF1-4, Tibia, and UMC cells as indicated. None of the HiPSCs formed colonies under such conditions except the CMC-derived cells. These experiments were performed in duplicate and repeated three times. B, representative HE staining of a teratocarcinoma arising from cells derived from the CMC colonies. The tumors, which were pathologically determined as teratocarcinomas, were collected from SCID mice 7 weeks after inoculation. Scale bar represents 100 μm. C, representative immunohistochemical staining for OCT4 in the CMC colony-derived teratocarcinoma. OCT4 is highly expressed in the tumors. Scale bar represents 100 μm.

Table 1. Teratoma-forming ability of HiPSCs in SCID mice

Cell line	Tumor incidence after subcutaneous cell transplantation (10 weeks)	
	3×10^6 cells	1×10^6 cells
CMC	3/4	1/5
hNF1-4	1/3	1/5
Tibia	1/3	0/5
UMC	1/3	0/5

HiPSCs, human induced pluripotent stem cells; SCID mice, severe combined immunodeficient mice. All data are presented as the number of mice bearing tumors / the number of mice underwent cell transplantation.

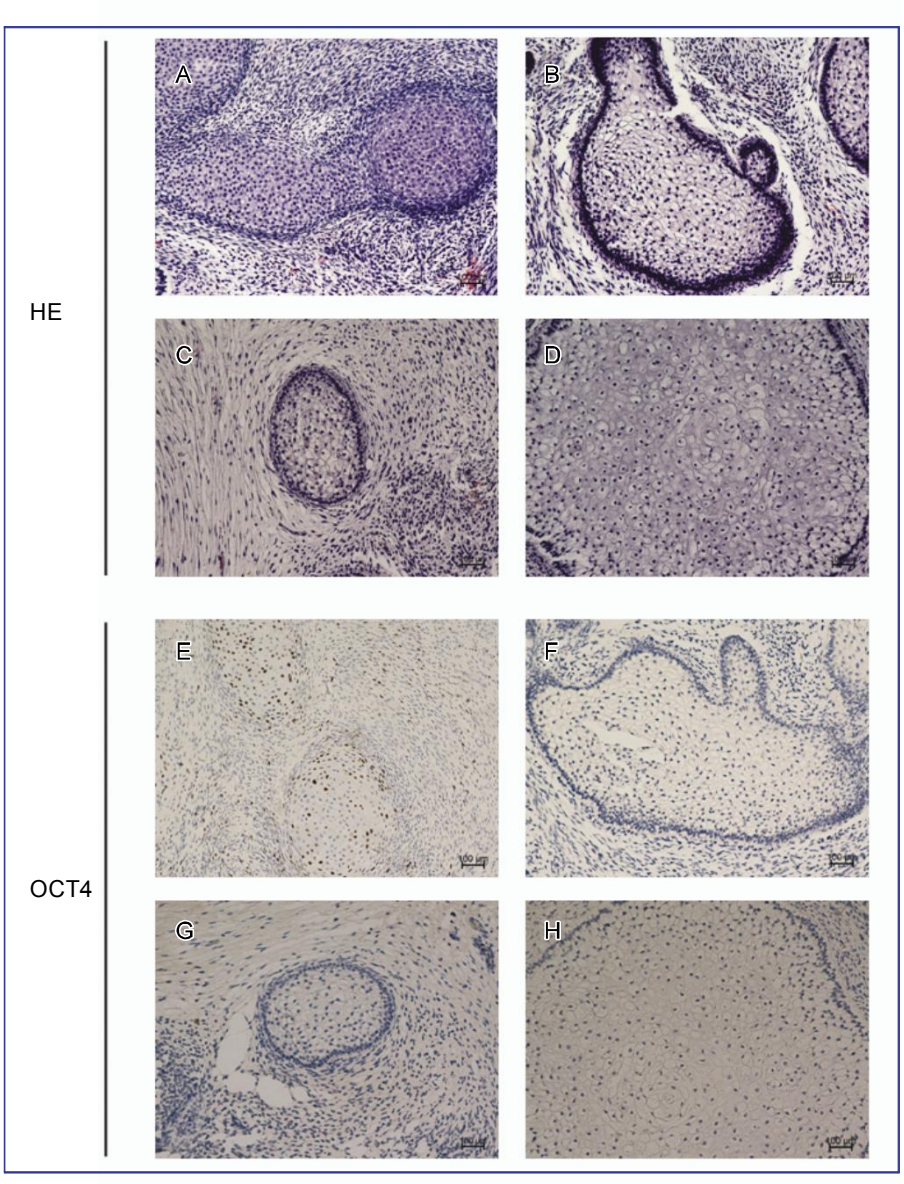

Figure 2 **HiPSCs produce teratomas/ teratocarcinomas in SCID mice.** A–D, representative HE staining of teratomas/ teratocarcinomas produced from inoculation with 3×10^6 CMC (A), hNF1–4 (B), Tibia (C), and UMC (D) cells after 10 weeks. CMC cells produced teratocarcinomas, whereas all other HiPSC lines formed teratomas *in vivo*. Scale bars represent 100 μm. E–H, representative immunohistochemical staining of OCT4 in teratomas/ teratocarcinomas derived from CMC (E), hNF1-4 (F), Tibia (G), and UMC (H). None of the tumors derived from HiPSCs, except CMC cells, were OCT4-positive. Scale bars represent 100 μm.

evaluate the extent of genomic instability in HiPSCs. For comparative purposes, we detected the baseline CNV in the parental lines (Table 2) using the same method. Specifically, we identified regions of DNA

amplifications/deletions in the genomes of HiPSCs (Table 2) and approximately 1 to 2 background CNV loci in the CMC and UMC parental cell lines (Table 3).

We compared the number of post-reprogramming CNVs in the four HiPSC lines to their respective parental cells (Table 3). The results suggested an association between tumorigenicity and the genomic instability induced by reprogramming. The CMC cell line, which is the most tumorigenic of our four HiPSC lines, harbored 6 CNV loci, more than any other cell line tested. Five of these CNV loci were created after cell reprogramming (Table 3). The hNF1-4 cell line, which produced teratomas in SCID mice with relatively few transplanted cells (1×10^6), had 4 CNV loci, all of which were induced by cell reprogramming (Table 3). The Tibia and UMC cell lines, which both gave rise to teratomas after inoculation with a relatively large number of cells (3×10^6), had only 1 CNV locus created after cell reprogramming (Table 3). These data indicate a plausible relationship between teratoma-forming ability, especially malignant potential, and the number of CNV loci, which is an indicator of genomic instability in HiPSC lines.

Among Yamanaka's factors (*OCT4*, *SOX2*, *C-MYC*, and *KLF4*), *C-MYC* and *KLF4* are well-known oncogenes. To further examine the relationship between tumorigenic potential and genomic instability, we studied the oncogenic H-RasV12–transformed NIH3T3 mouse embryonic fibroblast (MEF) cell line. Man *et al.*[20] had demonstrated that NIH3T3 cells became tumorigenic after the stable overexpression of the H-RasV12 oncoprotein. We used the CNV assay to estimate genomic instability caused by H-RasV12 overexpression. Consistent with our hypothesis, the NIH3T3-H-RasV12 cell line exhibited a large number of CNV loci (~2,760) compared to the parental NIH3T3 cells (Supplementary Figure S1 and Table S1).

Discussion

The discovery that somatic cells can be reprogrammed into iPSCs using defined factors has provided a promising future for the clinical application of

Table 2. Copy number variations (CNVs) in HiPSCs

HiPSC line	Copy number	Chromosome	Size (kb)	Parental cell line
CMC	1	1	217	Chorionic mesenchymal cells
	3	1	778	
	3	1	482	
	3	1	364	
	3	1	899	
	3	1	221	
hNF1-4	3	15	344	Adult cutaneous fibroblasts
	3	16	202	
	3	20	391	
	3	20	385	
UMC	1	1	217	Umbilical mesenchymal cells
	1	1	224	
	3	8	667	
Tibia	1	10	22,195	Periosteum mesenchymal cells

Table 3. The number of CNV loci detected before and after reprogramming in HiPSCs

HiPSC line	Number of CNV loci		Number of gained CNV loci after reprogramming
	Before reprogramming	After reprogramming	
CMC	1	6	5
hNF1-4	0	4	4
UMC	2	3	1
Tibia	0	1	1

personalized, stem cell-based therapies without the concern for immune rejection or ethical dilemmas. HiPSCs also provide a unique platform for studying genetic diseases *in vitro*[21]. However, there are concerns regarding the tumorigenicity and genomic instability of HiPSCs. In this study, we comprehensively explored the tumorigenic potential of our four previously established HiPSC lines. We determined that one of the HiPSC lines, derived from CMC cells, could both form tumorigenic colonies in cancer cell culture medium and generate malignant teratocarcinomas in SCID mice. Furthermore, based on the whole-genome CNV data, we identified a relationship between teratoma-forming ability and genomic instability in HiPSCs. In particular, the CMC cell line, among the four HiPSC lines, was the most vulnerable to CNV when compared with the other three cell lines. Due to the reprogramming process induced by the viral transfection of Yamanaka's factors (*OCT4*, *SOX2*, *C-MYC*, and *KLF4*), the diversity of genomic instability in those four HiPSC lines is most likely due to the tissue-specific characteristics of their parental cell lines.

Several reprogramming factors, including *C-MYC* and *KLF4*, are known for their oncogenic activities. This suggests the possible involvement of reprogramming factors in the induction of tumor formation in HiPSCs. Previous studies have indicated that the tumorigenic potential of mouse iPSCs in germline chimeras was correlated with overexpression of the *c-Myc* oncogene[6]. LIN28, a factor required for the reprogramming of human somatic cells[3], has been shown to facilitate transformation and play a role in germline malignancy formation[22,23]. Another important reprogramming factor, *SOX2*, exhibited lineage-survival oncogenic activity and was amplified in human lung/esophageal squamous cell carcinomas[24]. On the other hand, poorly differentiated, aggressive human tumors were found to express an embryonic stem cell-like gene signature[25]. Furthermore, cancer-related epigenetic abnormalities may arise during initial reprogramming and persist in HiPSC clones[26].

The above observations may support a role for "stemness"-inducing factors in tumorigenicity and malignancy. Hence, factors that may induce the tumorigenic potential of HiPSCs should be used with caution, or avoided, during the cell reprogramming process. C-MYC has been reported to be dispensable for the direct reprogramming of fibroblasts[27,28]. According to such results, although malignant transformation was a rare event (one out of four cell lines) in HiPSCs, oncogenic potential should be strictly assessed before the clinical application of HiPSCs.

p53, an essential tumor suppressor gene, plays a well-documented role in the reprogramming process due to its ability to counteract reprogramming[11,29-32]. Mutant p53 is frequently detected in human tumors and often acquires capabilities that can lead to genomic instability[33] and promote tumorigenesis[34]. Mutant p53 has been shown to facilitate somatic cell reprogramming and augment the malignant potential of reprogrammed cells[9]. These findings suggest a possible linkage between genomic instability and tumorigenicity in iPSCs. Our results provide direct evidence for this connection. Indeed, the CMC cell line, which harbored the greatest number of CNV loci among our four HiPSC lines, had the greatest tumorigenic ability compared to the other cell lines. Several recent reports have suggested that genetic abnormalities, such as aneuploidy, partial duplication/deletion, and somatic coding mutations, frequently exist in HiPSC lines[13-15,35]. Thus, genomic integrity could be used as an important indicator for oncogenic potential in HiPSCs and should be examined before the use of HiPSCs in any clinical application.

Since the discovery of iPSCs by Yamanaka's group in 2006[1], a number of improvements have been made to reduce their tumorigenic potential, such as excluding the *C-MYC* proto-oncogene during iPSC generation[27,28,36-39], transfection using non-integrating vectors free of virus or transgene sequences[40-42], and the direct delivery of recombinant reprogramming proteins[43,44]. Efforts to make HiPSCs safer are certainly valuable; however, Mayshar *et al.*[13] did not observe a higher incidence of aneuploidy in HiPSC lines with viral integration compared to lines derived without viral integration. Additionally, Hussein *et al.*[15] determined that genetic abnormalities may inevitably arise during the reprogramming process. Based on the observations mentioned above and our findings here, a priority in the field of HiPSC induction must be to study novel and safe induction methods that retain genomic stability.

Conclusions

Our discovery that HiPSCs can be transformed into tumors due to their intrinsic genomic instability points to the significance of quality controls. These controls should include a detailed assessment of tumorigenicity and a high-resolution test for genomic integrity prior to the clinical application of HiPSCs. It is of utmost importance to develop safe approaches that do not induce malignant potential or genomic instability during the reprogramming process.

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