FULL LENGTH ARTICLE

Identification and characterization of the cellular subclones that contribute to the pathogenesis of mantle cell lymphoma

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Abstract Mantle cell lymphoma (MCL) is a B-cell malignancy with poor clinical outcome and undefined pathogenesis. Development of clinically relevant cellular models for MCL research is an urgent need. Our preliminary observations lead the development of two novel hypotheses that we tested in this study: 1. multicellular spheroid might be a unique growth mode of early-
**Stage cells in MCL; 2. MCL might be a polyclonal tumor.** We made the following original observations that have not been reported: First, we have provided a new experiment method for enriching MCL early-stage cells and characterized the spheroid mode of growth as a unique feature of early-stage MCL cells in cell line as well as in clinical samples. Second, we have established a clinically relevant cellular model of MCL, the JeKo-1-spheroid cell line, that was highly enriched in early-stage sub-clones. JeKo-1-spheroid cells and the spheroid growing cells enriched from MCL patients exhibited comparably enhanced tumorigenic abilities and similar biological features. Third, Immunophenotypic analysis has revealed that MCL may be derived from precursor-B(pre-B), immature-B and mature-B cells, not only the mature-B cells as WHO classified in 2016. Fourth, MCL may be a polyclonal disease composed of CD19+/IgM−, CD19+/IgM+, CD19+/IgM−/Igλ+ three sub-clones, of which the CD19+/IgM− sub-clone might be the dominant sub-clone with the strongest tumorigenic ability. Fifth, CD19+/IgM− that differentiates MCL and normal B cells may represent a new marker for MCL early detection, minor residual disease monitoring after therapies and prognosis.

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**Materials and methods**

**Cell line and patient sample**

The JeKo-1-parental and JeKo-1-spheroid cell lines were cultured and maintained in RPMI 1640 (HyClone) supplemented with 20% heat-inactivated foetal calf serum (Excell), 2 mM l-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Blood or bone marrow specimens from patients and healthy donors were obtained after informed consent, as approved by Southwest Medical University of Institutional Review Boards. Mononuclear cells were isolated from patients and normal specimens by standard Ficoll gradient methods, and were maintained in methylcellulose (Methocult H4435, Stem Cell Technologies). After three to five generations, the cells were transferred into the same medium as JeKo-1-parental cell lines.

**Side population assay**

The Hoechst 33342 staining procedure was based on the method described by Goodell et al.18 Hoechst 33342 staining was observed using a FACS a flow cytometer (Becon–Dickinson, USA).

**Flow Cytometry and sorting**

Single-cell suspension cells were incubated with the respective conjugated antibody for 15 min at 4 °C and analyzed with a BD LSR. IgM-APC (catalog: 551062) was from BD Biosciences. CD19-FITC (catalog: 11-0199), CD45-Alexa Fluor 700 (catalog: 56-9459), CD3-PerCP-Cy5.5 (catalog: 45-0037) and CD34-PE-Cyanine 7 (catalog: 25-0349) were from eBioscience. CD38- PerCP-Cy5.5 (catalog: B49199), TDT-PITC (catalog: IM3524), CD22-PE (catalog: IM1835U), CD5-PerCP-Cyanine 5.5 (catalog: B49191), CD19-ECD (catalog: 652804), CD10-PE (catalog: A07760) and CD34-ECD (catalog: IM2709U) were from Beckman Coulter.

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**Introduction**

The study of clonal evolution is one of the frontiers in hematologic cancer research. Majority of the work in this area has been conducted in leukemia research.1–3 A case of co-occurrence of Hodgkin lymphoma (HL) and MCL in the same patient suggests sub-clonal evolution of HL from MCL.4 Moreover, the heterogeneity within MCL tumors and resistance to therapies imply that the MCL tumors are comprised of the cells with different tumorigenic capacities.5 These observations suggest that MCL might be polyclonally-driven.6 However, existing experimental evidence fails to explain the complexities of clonal dynamics in hematologic cancer.7

While the majority of prior studies hypothesized the mature B cells as the origin of MCL based on the expression of CD19 and IgM,8–10 few studies have postulated otherwise that CD19-negative (CD19e) B cells are the initiating cells.11,17 Early studies employed differentiation markers to identify the mature and immature cell for common hematological diseases: CD138 for multiple myeloma (MM),12 CD19 for MCL11 and CD34 for acute leukemia (AL).13 More recent studies used whole-genome sequencing,2 array comparative genomic hybridization,6 and light chain research14 to study malignant progression of hematologic cancer. Studies on the pathogenesis of MCL were primarily driven.6 However, existing experimental evidence fails to explain the complexities of clonal dynamics in hematologic cancer.7

By establishing a cellular model enriched in early stage cells that closely mimic the clinical progression of MCL, we have generated experimental evidence supporting the polyclonal nature of MCL, a finding that has significant clinical implications.
Propidium iodide were from Sigma. The gating strategy for MCL cells were selected using CD19+/IgM−, CD19+/IgM+ and CD19+/IgM− cells (gate i: CD45+/PI−; gate ii: CD34+/CD3−; gate iii: CD19+/IgM−, CD19+/IgM+ and CD19+/IgM−). The sorting purity was greater than 99% in the majority of samples. All the fractions were isolated by fluorescence-activated cell sorting (Aria, Becton Dickinson, San Jose, CA).

Detection of stem cell- and B cell-associated markers by qRT-PCR

Total RNA was extracted with TRIZOL (Invitrogen) according to the manufacturer’s protocol. A standard RT-PCR was conducted using a PrimeScript RT Master Mix (Takara) according to the manufacturer’s instructions.

Colony formation assay (CFA)

Each sorted population was plated in 35-mm2 dishes with methycellulose in accordance with the manufacturer’s instructions. Cells were incubated for two weeks at 37 °C in a 5% CO2 incubator. The clonogenic spheroids that consisting of a minimum of 40 cells were counted under microscopy.

Xenotransplantation in NOD/SCID mice

All mice used in the study were obtained from the core facility of Experimental Animal Centre, as approved by Animal Care Committee. The JeKo-1-parental and JeKo-1-spheroid cells were injected intraperitoneally at doses of 106 cells per NOD/SCID mouse (n = 4, 27 days). Highly purified CD19+/IgM−, CD19+/IgM− and CD19+/IgM− cells from JeKo-1-spheroid (n = 3, 19 days) and pt4 (n = 4, 9 days) were injected intraperitoneally at two doses 105 and 106, respectively. Tumor-bearing mice were sacrificed when mice were moribund. The brain, thymus, sternum, heart, lung, liver, spleen, kidney and adrenal gland, stomach, intestines and pancreas were collected for hematoxylin and eosin (H&E) staining, and the spleens were used for immunohistochemistry.

Immunohistochemistry (IHC)

Tissue samples were fixed in 10% buffered formalin phosphate, stored in 70% ethanol, then followed by paraffin embedding. Samples were serially sectioned and stained with H&E, anti-human IgM (catalog: ab17104, Abcam), CD20 (catalog: Kit-0001, Maxim), CD79a (catalog: RMA-0552, Maxim), Ki-67 (catalog: ZA-0502, ZSGB), CCND1 (catalog: RMA-0541, Maxim) and PAX5 (catalog: AM0281, Ascend).

Statistical analysis

When two groups were compared, the Student’s t-test with Welch’s correction was used. By default, two-tailed tests were performed. *P < .05 was defined as statistically significant. **P < .0001 were considered highly statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0.

Results

Multicellular spheroid is a unique growth mode of early-stage cells in MCL and the establishment of the JeKo-1-spheroid cell line

We found that in the early passages (<10 passages), the JeKo-1-parental cells was made of primarily single cells. However, there were approximately 1% of cells grew as multicellular spheroids (Fig. 1A). FCM analysis revealed that the ratio of CD19− B cells was 0.34% ± 0.07% (Fig. 1B, C) in JeKo-1-parental cells. As the culture prolonged (10–20 passages), the majority of single cells gradually died (Fig. 1A, C) and the surviving cells formed irregular-shaped multicellular spheroids (Fig. 1A). However, around the twentieth generation, towards the end of the batch cell death, the irregular spheroids became regularly-sized. We evaluated the cell viability by the ratio of propidium iodide negative (PI−) using FCM for the different growth modes. Cell viability decreased from 94.32% ± 0.86% in single-cell suspension growth phase to 63.77% ± 1.90% in the irregular multicellular spheroid mode of growth, but was restored to 96.12% ± 0.78% in the stable spheroid mode of growth (>20th passage, Fig. 1C, D). When sorted CD19− and CD19+ cells were cultured in vitro, CD19− cells grew as single-cell suspension and died within 2–3 weeks, while the CD19+ cells retained high cell viability and grew slowly as small spheroid for 3–4 weeks (data not shown). We named this derivative cell line the "JeKo-1-spheroid". The JeKo-1-spheroid consisted mostly the spheroids with only a few single cells (Fig. 1A) and could be serially passaged more than 100 generations with efficient recovery after freezing in liquid nitrogen. To assess the clinical relevance, we harvested and isolated fresh MCL cells from two patients (pt1, pt2) and cultured them under the same condition of JeKo-1-parental cells. The primary cultures of MCL clinical samples were also dominated by spheroid growth (Fig. 1E). Maintenance of hematological cancer cell in single-cell suspension culture for biological studies is the standard practice. However, spheroid growth has been a characteristic feature of stem-like cells isolated from solid tumor in serum-free culture condition.19,20 These findings prompt us to hypothesize that: "spheroid growth is a unique feature of early-stage cells in MCL". To test this hypothesis and to identify the cellular make-up of the spheroids, we used FCM to measure CD19− content. The proportion of CD19− cells was 0.34% ± 0.07% in JeKo-1-parental cells, it increased to 88.97% ± 3.50% in JeKo-1-spheroid, a total of 261 times enrichment (Fig. 1B, C). Comparable level of enrichment of CD19− population in clinical samples was achieved (98.46% ± 0.74%, Fig. 1B).

We also conducted analysis of side population (SP) cells by FCM–based Hoechst 33342 staining.18 SP cells are enriched source of cancer-initiating cells with stem cell properties, which have been identified in solid tumors and hematopoietic malignancies.21 Similar to the enrichment of CD19− cell population in JeKo-1-spheroid, SP population was increased 108 times, from 0.11% ± 0.01% in JeKo-1-
parental cell to 11.94% ± 1.17% in JeKo-1-spheroid (Fig. 1F). The proportion of SP cells was decreased by verapamil (50 μM) in JeKo-1-parental and JeKo-1-spheroid were 0.07%, 8.44% respectively (Fig. 1F), confirming the specificity of the findings.

Collectively, these results demonstrate that multicellular spheroid is a unique growth mode of early-stage cells in MCL (Fig. 1A). The novel cell line, the JeKo-1-spheroid, was enriched of CD19 early-stage cells and SP cells (Fig. 1B, F).

**Immunophenotypic characterization of the JeKo-1-spheroid cell line and identification of the three distinct sub-clones**

We conducted immunophenotypic analysis of JeKo-1-spheroid by multi-color FCM in order to determine the origin of cells that involved in MCL pathogenesis. The expression of CD34, CD38, TDT, CD22, CD5, CD10 and IgM was evaluated in CD19 cells (Fig. 2A-B), and the expression profile was compared to that of normal B cell differentiation stage (Fig. 2C). The negative expression of CD22 indicated that B cell differentiation antigens were not expressed in CD19 cells. The absence of CD34, CD38 and TDT expression in CD19 cells excluded the cellular origin of normal hematopoietic stem cells, lymphoid common progenitor cells and progenitor B cells, thereby demonstrated that JeKo-1-spheroid culture didn’t contain these cells.

The expression of CD34, CD10 and CD5 was consistent with the immunophenotype of MCL (Fig. 2A, 2B). Further, the expression of CD34, CD38, IgM, TDT and CD10 (Fig. 2A-B) in JeKo-1-spheroid cells was consistent with the immunophenotype of pre-B, immature B and mature B cells (Fig. 2C), suggesting that MCL pathogenesis might not be limited to mature B cells. It may involve cells from multiple differentiation stages (Fig. 2C). We also found that...
CD19 is only expressed in the mature B cells in JeKo-1-spheroid, which are not consistent with the normal B cell differentiation. However, CD19 expression is gradually increased from pre-B to immature B and to mature B cell differentiation. Thus, the negative expression of CD19 in the early stage of B-cell may also be a sign of poor differentiation.

Finally, we identified the presence of the three sub-populations with the immunophenotype of CD19$^-$/IgM$^-$, CD19$^-$/IgM$^+$, and CD19$^+$/IgM$^+$, corresponding to pre-B, immature B and mature B cells, respectively (Fig. 2B, C).

**JeKo-1-spheroid cell line exhibited enhanced oncogenic potential**

The transcription of Oct2, bcl-9, bcl-2, bcl-11b, c-myc, CCND1, bcl-11a, bmi-1, sox2, CD133 and nanog was analyzed by qRT-PCR (Table 1). JeKo-1-spheroid cells displayed low expression of the mature B cell transcription regulatory factor Oct2 (Fig. 3A), corroborating the results from immunophenotyping analysis that JeKo-1-spheroid cell line was enriched for immature B cells. In contrast, JeKo-1-spheroid cells showed elevated expression of the

| Gene name | Forward primers | Reverse primers |
|-----------|----------------|-----------------|
| Bcl-9     | AGAGAGAAGCAGCACGGGCTTC | CTGCGATCTGTTATCTGGAAG |
| Bcl-2     | TTTGAGTTCGAGTGGTTGCCAT | TGACTTCAGTGGGTCAGG |
| Bmi 1     | TTGAGTTCGAGTGGTTGCCAT | GGTGCTGTTATCTGGAAG |
| Bcl-11b   | ACTGAAGCAGCACGGGCTTC | ATGAGTTCAGTGGTTGCCAT |
| Nanog     | AGATGCCCTCACAGGAGACCT | TTGAGTTCGAGTGGTTGCCAT |
| CD133     | GCTTTGCAAATTCCTGTTGCC | TTGAGTTCGAGTGGTTGCCAT |
| C-myc     | TTTGAGTTCGAGTGGTTGCCAT | CAGCAGTGGTGAATCTG |
| Cyclin d1 | GATGAGGAAAGTGGCAGCACGAC | GATGAGGAAAGTGGCAGCACGAC |
| Sox 2     | CACCCAGAGGATCTCCAGGAA | TGTATGGGTTGGAATCTG |
| Bcl-11a   | ACAAAGGAAAATACGGAATG | TTTGAGATATGTTGGAATCTG |
| Oct 2     | GAGGAAAAGTTGGAATGGAATG | GAGGAAAAGTTGGAATGGAATG |
| β-actin   | AAGGAAAAGTTGGAATGGAATG | GAGGAAAAGTTGGAATGGAATG |
Figure 3  Tumorigenic potential of JeKo-1-spheroid and JeKo-1-parental cells. (A) mRNA expression of stem-cell, oncogene and B-cell differentiation markers in JeKo-1-spheroid and JeKo-1-parental cells. (B) Quantitation of CFA by JeKo-1-spheroid and JeKo-1-parental. **: P < .0001, using the single variance analysis method. (C) H&E staining and IHC of JeKo-1-spheroid or JeKo-1-parental-derived tumour xenografts; bar = 50um. (D) Representative images of the stomach and spleen from mice with JeKo-
oncogenes and stem cell markers including bcl-9, bcl-2, bcl-11b, sox2, CD133, c-myc, CCND1 and bmi-1, consistent with previous report.25,26 and low expression of bcl-11a and nanog (Fig. 3A). Overall, our results showed that JeKo-1-spheroid cells expressed higher levels of oncogenes and stem cell markers in comparison to the JeKo-1-parental cell line.

In order to confirm that JeKo-1-spheroid cells has higher clonogenic potential than that of JeKo-1-parental cells, we conducted CFA in vitro and xenotransplantation assays in vivo. The oncogenic potency in vivo was assessed by: (1) the number of tumor organs; (2) H&E assessment of bleeding and infection organ, determines the effect of MCL tumor growth on normal hematopoiesis and immune system. In comparison to the JeKo-1-parental cells, the JeKo-1-spheroid cells formed a significantly higher number of colonies in vitro (Fig. 3B).

Xenograft tumors from the two cell lines expressed comparable levels of CD79a, CD20, Pax5, IgM, CCND1 and Ki-67 proliferation antigens (Fig. 3C).5 The tumor-invaded stomach of one mice in JeKo-1-spheroid group was significantly smaller than the normal stomach in JeKo-1-parental group (Fig. 3D). The spleen that had tumor infiltration of one mice in JeKo-1-spheroid group was significantly larger than that in JeKo-1-parental group (Fig. 3D) with extra-medullary hematopoiesis (data not shown), a biological process in order to restore hematopoiesis when bone marrow compensatory function is inadequate. In contrast with normal cardiopulmonary pathology in the JeKo-1-parental group, two mice in the JeKo-1-spheroid group developed pulmonary infection and bleeding with visible purulent secretion of the red surface (Fig. 3E), with a large number of erythrocytes and neutrophils by H&E staining (Fig. 3F). The pericardial infection in the JeKo-1-spheroid group formed an abscess in the heart that caused the cardiopulmonary adhesion (Fig. 3E). Intestinal infections in the JeKo-1-spheroid group made the segment of intestine lose its healthy pale pink color, and formed yellow encapsulated abscesses (Fig. 3E). However, none cardiopulmonary infection and hemorrhage occurred in JeKo-1-parental group (Fig. 3E). The tabulated data showed that in the JeKo-1-spheroid group, the tumor had spread to eleven organs including the spleens, but in the JeKo-1-parental group, only spleen was involved without signs of wide disseminated tumor (Fig. 3G; Table 2). There were seven hemorrhagic and infectious organs in JeKo-1-spheroid group, in particular, two mice had serious heart and lung infections and hemorrhage, and one mouse had a severe gastrointestinal infection. In comparison, JeKo-1-parental group had infections only in two organs and had no organ hemorrhage (Table 2).

In sum, we found that the JeKo-1-spheroid cells exhibited enhanced tumor-forming abilities in comparison to the JeKo-1-parental cells, that was accompanied by dissemination, severe and extensive complications of infection and hemorrhage which recapitulated the clinical course of MCL progression.

### Comparison the oncogenic potential of CD19/IgM sub-populations and the polyclone pathogenesis of MCL

We subsequently compared the oncogenicity of three sub-populations we identified in JeKo-1-spheroid cells. The comparative analysis was also performed with sub-populations derived from four patients with stage IV MCL (pt1, pt2, pt3 and pt4) and one healthy donor. We conducted CFA in vitro and used three sub-populations of pt4 and JeKo-1-spheroid cells in the xenotransplantation assay in vivo.

FCM analysis revealed that the healthy donor samples expressed CD19<sup>e</sup>/IgM<sup>e</sup>, CD19<sup>+</sup> /IgM<sup>e</sup>, CD19<sup>e</sup>/IgM<sup>−</sup> and CD19<sup>+</sup> /IgM<sup>−</sup> four sub-populations (Fig. 4A; Table 3). In contrast, the CD19<sup>+</sup> /IgM<sup>−</sup> sub-population was absent in all four clinical specimens and the JeKo-1-spheroid cells (Fig. 4A; Table 3). The PBMCs of pt4 were collected from a patient with a large cell variant of MCL showing leukemic conversion which is the terminal stage of MCL. Whereas the other three clinical samples were BMMCs. CD19<sup>−</sup> proportion in pt4 sample was as high as 99%, suggesting the importance of CD19<sup>−</sup> early-stage clones in MCL disease progression.

CFA analysis demonstrated that all four CD19/IgM sub-populations from one healthy donor were unable to form colonies (data not shown). In contrast, the three sub-populations from pt1, pt2, pt4 and JeKo-1-spheroid were all able to form colonies in vitro (Figs. 4B, C and 5A—D). In summary, the three sub-populations (henceforth called "sub-clones") in MCL are capable of forming colonies with the relative potency scored in the order as following: CD19<sup>−</sup>/IgM<sup>−</sup> > CD19<sup>−</sup>/IgM<sup>e</sup> > CD19<sup>+</sup> /IgM<sup>−</sup> (Fig. 4B, C).

We analyzed xenograft tumors by IHC for the expression of CCND1, CD20, CD79a, PAX 5, Ki-67 and IgM. The three

| Cell               | # of mice with tumours | # of tumor-infiltrated organs* | # of infected organs* | # of hemorrhagic organs |
|--------------------|------------------------|-------------------------------|-----------------------|-------------------------|
| JeKo-1-parental    | 4/4                    | 4/4                           | 2/44                  | 0/44                    |
| JeKo-1-spheroid    | 4/4                    | 11/4                          | 7/44                  | 7/44                    |

1spheroid or JeKo-1-parental-derived tumour xenografts. (E) Representative images of organs from mice inoculated with JeKo-1-spheroid and JeKo-1-parental. Red arrows indicate the infected areas from JeKo-1-spheroid (upper panel): pulmonary and heart (i), intestine (ii); normal organs from JeKo-1-parental (lower panel): pulmonary and heart (iii), intestine (iv). (F) H&E staining of pulmonary infection and hemorrhage from mice inoculated with JeKo-1-spheroid; bar = 50um. (G) Morphological features of the JeKo-1-spheroid and JeKo-1-parental-derived tumour xenografts; red arrows indicate the tumor lesions from JeKo-1-spheroid (upper panel), the normal organs from JeKo-1-parental (lower panel); bar = 50um.
sub-clones isolated from JeKo-1-spheroid and pt 4 sample formed tumor masses only in the spleen (Figs. 5A, D and 6A, B), no other organs were involved (Table 4). Nevertheless, the spleen showed the high expression of the genetic hallmark of MCL, CCND1 (Fig. 6A, B), similar to the high CCND1 expression observed in xenograft tumors derived from the polyclone in JeKo-1-spheroid (Fig. 3C). Xenograft tumors from CD19+/IgM+ and CD19+/IgM+/IgM+ monoclonal from JeKo-1-spheroid and pt4 samples didn’t showed comparable levels of CD20, CD79a, PAX 5, Ki-67 and IgM (Fig. 6C) markers expressions in comparison to the polyclone in JeKo-1-spheroid (Fig. 3C). However, xenograft tumors from monoclones in JeKo-1-spheroid and pt 4 sample, and from the polyclone in JeKo-1-spheroid all exhibited similar pathological characteristics of MCL, in particular, the presence of small to medium sized lymphoid cells with irregular nuclear contours (Fig. 6A–C).27

Together, these data demonstrate that while the monoclones retained CCND1-specific high expression, the expression of CD20, CD79a, PAX 5, Ki-67 and IgM couldn’t be detected (Fig. 6A–C) and without signs of disseminated tumor. Furthermore, none of the monoclon from JeKo-1-
spheroid and pt4 was capable of generating xenograft tumors that fully recapitulate the heterogeneity of polyclone tumors in JeKo-1-spheroid judged by the total number of organs involved, immune phenotypes and the oncogenic capability (Figs. 3C,D,G and 6A–C; Tables 2 and 4). We found that the synergistic tumorigenic capability of three sub-clones is better than that of any monoclon.

We observed that one mice in CD19⁺/IgM⁺ group (cell inoculation dose: 10⁴/mouse) from JeKo-1-spheroid had two infected organs and three hemorrhagic organs (Fig. 6D,E; Table 4). One mice in the CD19⁺/IgM⁺ group (cell inoculation dose: 10⁴/mouse) from JeKo-1-spheroid had one infected organ and one hemorrhagic organ (Table 4). However, no mice in CD19⁺/IgM⁺ group from JeKo-1-spheroid was infected and bleeding (cell inoculation dose: 10²/mice and 10⁴/mice, Table 4). In contrast, two mice in CD19⁺/IgM⁺ from pt4 group at 10⁷ and 10⁸ doses of tumor cell inoculation had two and six infected and hemorrhagic organs, respectively (Fig. 6D,E; Table 4). Two mice in CD19⁺/IgM⁺ from pt4 group at 10⁷ and 10⁸ doses of tumor cell inoculation had two and three infected and hemorrhagic organs, respectively (Fig. 6D,E; Table 4). However, no infected and hemorrhagic organs were observed in CD19⁺/IgM⁺ group from pt4 group (cell inoculation dose: 10²/mouse) (Table 4). Two mice in CD19⁺/IgM⁺ group (cell inoculation dose: 10⁴/mouse) from pt4 group, a total of five

### Table 3  Comparison the expression of CD19/IgM sub-populations in JeKo-1-spheroid, four patients and healthy donor.

| Cells           | CD19⁺/IgM⁻ (%) | CD19⁺/IgM⁺ (%) | CD19⁺/IgM⁺ (%) | CD19⁺/IgM⁺ (%) |
|-----------------|----------------|----------------|----------------|----------------|
| Pt1⁺            | 9.04           | 74.37          | 16.51          | 0              |
| Pt2⁺            | 11.69          | 76.63          | 11.18          | 0              |
| Pt3⁺            | 2.74           | 77.23          | 19.95          | 0              |
| Pt4⁺            | 94.31          | 4.63           | 0.59           | 0              |
| JeKo-1-spheroid | 7.70 ± 3.45    | 76.91 ± 7.27   | 15.00 ± 6.14   | 0              |
| Normal⁺         | 52.44          | 11.71          | 11.93          | 23.92          |

* a BMNCs from patients with clinically confirmed stage IV MCL.

* b PBMCs from a patient with a large cell variant of MCL showing leukemic conversion and one healthy donor.

![Figure 5](image_url)  Sorting strategy of three sub-clones derived from patients and JeKo-1-spheroid cell line. Isolation of CD19⁺/IgM⁻, CD19⁺/IgM⁺ and CD19⁺/IgM⁺ sub-clones from the JeKo-1-spheroid cell line (A), pt1 (B), pt2 (C) and pt4 (D).
organs were infected and hemorrhagic (Fig. 6E; Table 4). Thus, the CD19⁺/IgM⁺ sub-clone from either JeKo-1-spheroid or pt4 was more likely to develop widespread organ infection and bleeding when compared to the other monoclonal sub-clone from either JeKo-1-spheroid (i) and pt 4 (ii). (D) Haemorrhagic and infection manifestations induced by the CD19⁺/IgM⁺ subclone in NOD/SCID mice. Yellow arrows indicate the normal kidneys (i), red arrows indicate the haemorrhagic kidneys in JeKo-1-spheroid (ii), blood can be seen in the stomach from pt4 (iii), and infectious intestine was yellow and formed yellow encapsulated abscesses in pt4 (iv). (E) H&E analysis of pulmonary infection and hemorrhage from the three subclones in pt 4: CD19⁻/IgM⁻ (i); CD19⁻/IgM⁺ (ii); CD19⁺/IgM⁺ (iii).

**Discussion**

Since worldwide recognition of MCL in 1994, it is known for its dismal prognosis, with a median overall survival (OS) rate of 3 years. The lack of clinically relevant cellular models and effective research methods have hindered in-depth mechanistic investigation of MCL.

It is widely believed that blood cells grow as single cell suspension in vitro. However, spheroid growth has been a characteristic feature of stem-like cells isolated from solid tumor in serum-free culture condition. In this report, we demonstrate that growth as multicellular spheroid is a unique feature of early-stage cells in MCL. In contrast to multicellular spheroid growth of non-blood cancer stem cell that requires the switch of serum culture medium into serum free medium, spheroid growth of MCL can be maintained in the same culture condition that contains serum.

The JeKo-1-parental cell line, established from PBMCs of a terminal stage MCL patient with a large cell variant of...
MCL showing leukemic conversion, similar to pt4 used in our study.16 Interestingly, when the JeKo-1-parental cell line was originally cultured in vitro, multicellular spheroid mode of growth was documented. After the first two months, the established cell line adopted the single-cell suspension growth.16 This observations indicate that when MCL cells were initially isolated from patient, their growth in vitro retained the cluster aggregation feature. Single-cell suspension growth maybe a gradual adaptive feature of long-term culture in vitro, retaining a very low proportion of early immature cells B cells via multicellular spheroid growth. Differentiation of progenitor B cells is regulated by mature B cells in vivo, as decrease in the number of mature B cells in the peripheral blood can stimulate early B progenitor differentiation.28 This theory consistent with our observation that the death of a large number of single mature B cells stimulated the growth and enrichment of early-stage B cells with spheroid growth (Fig. 1A, C). We speculate that under similar condition, early-stage cells of other hematopoietic tumors could also be enriched by spheroid growth. Future studies will explore whether this experimental approach can be used to establish the clinically relevant cellular models of other hematologic cancers for the study of clonal evolution and heterogeneity.

The various features of JeKo-1-spheroid such as the spheroid growth mode, enrichment of CD19+ and SP cells, high expression of oncogenes and stem cell markers, enhanced tumorigenic and metastatic capability in vivo, high incidence of infection and bleeding better recapitulate the clinical features of MCL. Employment of the JeKo-1-spheroid cell line model yielded a set of original findings:

First, MCL might be a polyclonal disease, and CD19+/IgM− might be the dominant sub-clone. The three sub-clones (CD19+/IgM+, CD19+/IgM−, CD19−/IgM+) were present in both JeKo-1-spheroid cells and MCL clinical samples, among which the CD19+/IgM− sub-clone is the dominant clone. Further, the monoclonal tumors retaining only CCND1 expression and mantle zone growth pattern feature of MCL in vivo, which consistent with in situ mantle cell neoplasia (ISMCN).10,29 ISMCN is a very indolent tumor and may not require therapeutic intervention. The monoclonal tumors were incapable of manifesting the full pathological phenotypes of clinical MCL when compared to the polyclonal tumors of JeKo-1-spheroid cell line. Our results are similar to those of breast cancer studies,7 that none of the monoclonal were metastatic. This finding indicates that CCND1 high expression is necessary at the initiation of MCL, but not sufficient to attain the full scope of pathologic phenotypes.1,30

Second, CD19+/IgM− that differentiates MCL and normal B cells may represent a new clinical marker. We found that the CD19+/IgM− subgroup was only present in normal B cells (Fig. 4A; Table 3). Expanded clinical cohorts are required to confirm this finding.

Third, CD19+/IgM− that differentiates MCL and normal B cells may represent a new clinical marker. We found that the CD19+/IgM− subgroup was only present in normal B cells (Fig. 4A; Table 3). Expanded clinical cohorts are required to validate the diagnostic and prognostic importance of CD19+/IgM−.

In summary, our work provides a method for the enrichment of early-stage cells and experimental basis for the polyclonal nature of MCL pathogenesis which may yield improved diagnosis and prognosis, as well as a new path for developing more effective treatment for MCL. We plan to initiate new studies directed at investigating mechanisms regulating clonal evolution and the chemo-resistant of the sub-clones. This will ultimately lead to improved outcomes for MCL patients.

### Conflicts of interest

The authors declare no potential conflict of interest.

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