α-thalassaemia combined with hereditary spherocytosis in the same patient

XIAOHONG LI1, LIN LIAO1, XUELIAN DENG1, JIAN HUANG1, ZENGFU DENG2, HONGYING WEI3, WUNING MO1 and FAQUAN LIN1

1Department of Clinical Laboratory, First Affiliated Hospital of Guangxi Medical University; 2Department of Clinical Laboratory, First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine; 3Department of Paediatrics, First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China

Received August 23, 2017; Accepted November 15, 2017

DOI: 10.3892/etm.2017.5579

Abstract. A family of four from the Guangxi Zhuang Autonomous Region of China, including a child with α-thalassaemia and hereditary spherocytosis (HS), underwent laboratory identification, and genetic analysis. After harvesting peripheral blood samples from the child patient and his family members, GAP-polymerase chain reaction (PCR) and reverse dot-blot tests were used to identify thalassaemia genotypes. After amplifying exons and the adjacent introns of solute carrier family 4 member 1 (Diego blood group) (SLC4A1), ankyrin 1, spectrin α erythrocytic 1, spectrin β erythrocytic and erythrocyte membrane protein band 4.2 by PCR, DNA sequencing was utilised to detect gene mutations of HS. The thalassaemia gene of the child patient was -α3.7/αα and identical to the genotype of his mother. DNA testing of HS identified two mutation sites on the SLC4A1 gene: Exon 3 c.113A>C (Asp 38 Ala) and intron 7 c.609+86G>A. The father and older sister of the patient also had the same mutations. Due to the mutual interference with disorders of haemoglobin synthesis and erythrocyte membrane defects of laboratory results, it is difficult to diagnose HS when it coexists with thalassaemia. When clinical manifestations and laboratory results cannot be explained by a single haemolytic anaemia, the possibility of combining with another haemolytic anaemia should be considered. Thus, it is necessary to perform pedigree investigation and genetic analyses for a final diagnosis.

Introduction

Thalassaemia is an autosomal recessive inherited disease characterised by a deficiency or deletion in the synthesis of α and β globin chains. There are various ethnic and regional differences in China. Thalassaemia mainly occurs in the southern part of China especially in the Guangdong Province and Guangxi Zhuang Autonomous Region of China (1,2). A previous study (3) showed that the total heterozygous frequency of haemoglobinopathies was 24.51%. Of these, α-thalassaemia accounted for 17.55% and β-thalassaemia accounted for 6.43%. Li et al (4) reported that in 356 cases of hereditary haemolytic anaemia, constituent ratios of aetiology were 42.56% in erythrocyte membrane disorders, 34.62% in haemoglobinopathies, and 22.82% in erythrocyte enzymopathies. Hereditary spherocytosis (HS) is the most common hereditary haemolytic disease and is caused by changes to erythrocyte membranes. Approximately 75% of patients with HS have autosomal dominant inheritance, and the remaining 25% patients have no family history of spherocytosis, the presence of spontaneous mutation or recessive inheritance. Typical clinical manifestations of moderate and severe HS are anaemia, jaundice, and splenomegaly. Clinical manifestations of mild HS are not typical and can even be completely asymptomatic (5). To the best of our knowledge, the coexistence of α-thalassaemia and HS in an individual is seldom reported, and no such study has been reported mutations in both α-thalassaemia and solute carrier family 4 member 1 (Diego blood group) (SLC4A1) genes from China. We herein describe the laboratory test results of a child patient with α-thalassaemia and HS and his family members.
Patients and methods

Clinical history. The case was a 5-year-old premature male infant (the second child of five pregnancies) in the Guangxi Zhuang Autonomous Region of China. He was delivered by caesarean delivery at a local hospital due to ‘intrauterine distress’. At birth, the patient experienced a pale face and yellow skin without an obvious cause of the symptoms, and was taken to a local hospital. Routine blood examination revealed an erythrocyte count of 1.24x10^12/l, haemoglobin (Hb) of 48.0 g/l, hematocrit of 0.16, reticulocyte percentage of 58.21%, and immature reticulocyte ratio of 35.2%. Coombs test was negative. Glucose-6-phosphate dehydrogenase (G6PD) activity was normal. Total bilirubin was 226.9 µmol/l and indirect bilirubin was 201.6 µmol/l. No abnormalities were observed by other laboratory tests. Bone marrow examination exhibited active bone marrow hyperplasia. The patient was diagnosed as haemolytic anaemia. He was discharged after his symptoms were reduced by blood transfusion. Because his Hb levels decreased to 40.0-60.0 g/l approximately every 4 months, he received blood transfusions in a local hospital. Following suspected thalassaemia, he attended our hospital. Physical examination showed that the sclera and whole body were slightly yellow, the liver was not palpable, but the spleen could be felt 2.5 cm under the ribs. Cardiopulmonary examination did not show abnormalities and ascites was not detected. Erythrocyte parameters revealed microcytic hypochromic anaemia. HbA2 was 0.025, HbF was 0.011, serum ferritin was 1,467.37 ng/l, and iron saturation was 0.84. Three haemolysis tests and an isopropanol test were negative. A heat instability test showed 1.00%. Degenerated globin bodies were 0.20%, plasma free Hb was 61.6 mg/l, and plasma haptoglobin was <0.125 g/l. Glucose phosphate isomerase activity was normal. No abnormalities were determined by other laboratory tests.

Pedigree investigation. The father was 38 years of age, the mother was 39 years, and his older sister was 8 years old. Both his parents were from Guangxi Zhuang nationality, and they had a non-consanguineous marriage, were healthy, and without family medical history. To identify the cause of anaemia further, haematological examination, thalassaemia genetic testing, and HS gene mutation analyses were conducted in the patient, his parents and older sister.

Ethical approval. All participants signed informed consent forms prior to participation. This study was approved by the Ethics Committee in Research of the First Affiliated Hospital of Guangxi Medical University.

Haematological examination. After harvesting 5 ml of venous blood (EDTA-K2 anticoagulation), routine blood tests and reticulocyte detection were performed with a Beckman Coulter LH780 haematology analyser (Beckman Coulter, Inc., Brea, CA, USA). Hb electrophoresis was performed in strict accordance with the instructions of matched reagents (Helena Laboratories, Beaumont, TX, USA). HbA2 and HbF were quantitatively determined. Erythrocyte membrane protein was extracted and analysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Haemolysis testing was conducted in accordance with the National Guide to Clinical Laboratory Procedures (6).

Genetic analysis. DNA was extracted with whole blood genomic DNA extraction reagents (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. Three common deletions of α-thalassaemia (-SEA, -α3.7, and -α4.2) were determined by GAP-polymerase chain reaction (PCR) with the reagents provided by Shenzhen Yishengtang Biotechnology Co., Ltd. (Shenzhen, China). Three point mutations of α-thalassaemia gene (HbCS, HbQS, and Hb West) and 17 kinds of β-thalassaemia gene were detected by reverse dot-blot tests. Primers were designed for the exons of SLC4A1, EPB42, ANK1, SPTA1 and SPTB and their adjacent intron sequences, and were synthesised by Sangon Biotech Co., Ltd. (Shanghai, China). PCR reaction systems included 25 µl of 2X Taq PCR Master Mix (Takara Biotechnology Co., Ltd., Dalian, China), 2 µl of genomic DNA, 1 µl of upstream primer, and 1 µl of downstream primer. ddH2O was added to a total volume of 50 ml. Reaction conditions were as follows: Initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at an optimal temperature (optimal annealing temperature was different for different fragments of different genes) for 35 sec, extension at 72°C for 45 sec, followed by extension at 72°C for 5 min. Samples were stored at 4°C until use. PCR products were purified, retrieved, and unidirectionally sequenced by Sangon Biotech Co., Ltd. The sequencing results were compared with the standard sequences in the National Centre for Biotechnology Information. Each sample with a questionable sequencing result was amplified by PCR and verified by bidirectional sequencing.

Results

Laboratory results. Laboratory results of the patient and his family members are shown in Table I.

Peripheral blood smears. Peripheral blood smears of the patient showed mature erythrocytes varied slightly in size and had weak staining in some areas. Some cells were spherical or target-shaped, and polychromatic erythrocytes were observed. No noticeable abnormalities were observed in the peripheral blood smears of the patient's parents or older sister (Fig. 1). Haematological examination revealed a mean corpuscular volume (MCV) <80 fl and mean corpuscular haemoglobin (MCH) <27 pg in the child patient, suggesting microcytic hypochromic anaemia (7).

Genetic analysis of thalassaemia and HS. Genetic diagnosis results demonstrated that the α-thalassaemia gene of the child patient was -α3.7/αα, and identical to the genotype of his mother. No abnormalities were determined in β-thalassaemia gene. The genetic diagnosis of his father and older sister did not show abnormalities. DNA sequencing showed SLC4A1 gene had two mutation sites: Exon 3 c.113A>C and intron 7 c.609+86G>A in the proband. In exon 3 c.113 A>C of SLC4A1, the GAC codon was mutated to GCC, leading to an Asp 38 Ala missense mutation. Mutations were not detected in ANK1, SPTA1, SPTB or EPB42. His father and older sister had the same mutations (Figs. 2 and 3).
α-thalassaemia is a defect or deficiency of α-globin chain synthesis in Hb caused by α-globin gene defects. This results in fewer α-globin chains being produced and decreased Hb synthesis, and leading to microcytic hypochromic anaemia. A relative excess of β/γ globin chain polymerizes and deposits on erythrocyte membranes, which induces oxygen free radical reactions, and decreases erythrocyte deformability and mechanical stability. This causes haemolysis and ineffective haematoopoiesis. In accordance with the clinical manifestations and laboratory findings [reduced Hb, MCV, MCH, and MCH concentration (MCHC); normal or diminished HbA₂; normal iron metabolism; peripheral blood smears exhibit targeted erythrocytes and erythrocyte debris], combined with a family history and epidemiology of the patient, the patient was preliminarily diagnosed with α-thalassaemia. Genetic analysis confirmed the diagnosis of α-thalassaemia (8-10).

In the present study, the α-thalassaemia gene of the child patient was -α3.7/αα, which is a α-thalassaemia carrier and has no clinical symptoms or signs. However, the patient had suffered from significant anaemia and jaundice since birth. Erythrocyte osmotic fragility was not obviously altered in the patient. MCV, mean sphered corpuscular volume (MSCV), MCH and mean reticulocyte volume (MRV) were remarkably reduced, MCHC was normal, whereas red blood cell distribution width (RDW) was dramatically increased. Coombs test was negative. PK and G6PD activities were normal. These findings indicated that this patient did not suffer from α-thalassaemia alone. When these symptoms were combined with data from the peripheral blood smears, we considered he might also suffer from HS.

HS is a hereditary haemolytic disease where erythrocyte membrane defects cause erythrocyte destruction. Membrane protein defects can lead to pathophysiological changes including a weakened vertical connecting force between the membrane skeleton and the membrane leading to bilayer lipid instability, and vesicle budding and loss. As the erythrocyte surface area is reduced, the surface area and volume ratio also decrease, causing the formation of small spherical erythrocytes. Decreased erythrocyte membrane protein phosphorylation, increased catalase and membrane-bound Hb, and a reduced surface area of the membrane lead to a decrease in erythrocyte deformability; therefore, erythrocytes are easily destroyed in the spleen resulting in hemolysis (11). Typical clinical manifestations of HS are anaemia, splenomegaly, and jaundice, often accompanied by gallstones. Patients with typical clinical manifestations according to their family history and laboratory tests (increased MCHC and Ret, peripheral blood smears with an increased number of spherical erythrocytes) can be diagnosed as HS without other tests. However, the clinical manifestations of HS are heterogeneous. For patients with atypical clinical manifestations, a cold haemolysis test, eosin-5-maleimide binding test, and gel electrophoresis can be conducted. At present, SDS-PAGE is commonly used to analyse membrane proteins, however it is not sensitive to HS patients or asymptomatic patients. Compared with SDS-PAGE, quantitative fluorescence real-time PCR has a higher sensitivity and specificity, and direct sequencing can identify mutation sites of HS after extracting genomic DNA and conducting PCR (12,13).

### Discussion

The coexistence of thalassaemia and HS in the same patient is seldom reported (14-17). Because the two different erythrocytes defects will interfere with the relevant laboratory results, α-thalassaemia combined with HS is easily misdiagnosed or missed. Akar and Gökçe (16) reported that 13 patients with combined HS and β-thalassaemia from 10 families. Compared to patients with HS alone, MCHC was increased, but MCV and MCH were obviously diminished in patients with combined HS and β-thalassaemia. Study of HS by Broséus et al (18) confirmed that when MCV-MSCV >9.6 fl, the diagnostic sensitivity to HS was 100% and specificity was 90.57%. We performed a similar pre-study, and analysed MCV and MSCV in healthy control group, thalassaemia group and HS group. Our previous study (19) demonstrated that when MSCV <MCV, the diagnostic sensitivity to HS was 98.25% and specificity was 99.10%. Of note, MSCV was only higher than MCV in one patient with thalassaemia combined HS. In addition, we also verified that MRV was obviously lower in the HS group compared with the healthy control group, thalassaemia group, G6PD group and autoimmune haemolytic anaemia group. When MRV was >95.77 fl, the diagnostic sensitivity and specificity to HS were 86.80 and 91.20%, respectively (20). In this study, the MRV of our patient was 80.50 fl, which might have been affected by HS. A MSCV >MCV indicated that he experienced another disorder in addition to HS.

| Characteristic     | Patient | Father | Mother | Older sister |
|-------------------|---------|--------|--------|-------------|
| RBC (x10¹²/l)     | 3.16    | 4.96   | 5.46   | 4.82        |
| Hb (g/l)          | 71.60   | 155.40 | 143.20 | 126.00      |
| MCV (fl)          | 70.6    | 93.65  | 79.65  | 80.03       |
| MCH (pg)          | 22.66   | 31.31  | 26.20  | 26.13       |
| MCHC (g/l)        | 320.90  | 334.30 | 328.90 | 326.50      |
| MSCV (fl)         | 72.76   | 93.29  | 83.10  | 83.21       |
| MRV (fl)          | 80.50   | 110.11 | 105.07 | 100.06      |
| Ret (%)           | 0.004   | 0.008  | 0.023  | 0.013       |
| RDW               | 0.21    | 0.13   | 0.15   | 0.13        |
| TBIL (μmol/l)     | 46.10   | 15.40  | 32.00  | 27.90       |
| IBIL (μmol/l)     | 38.00   | 3.90   | 16.70  | 7.20        |
| AGLT₉₀ (sec)      | >290    | >290   | >290   | >290        |
| Coombs test       | -       | -      | -      | -           |
| PK                | N       | N      | N      | N           |
| G6PD              | N       | N      | N      | N           |
| Haemoglobin       | N       | N      | N      | N           |
| electrophoresis   | SDS-PAGE| N     | N      | N           |

*, negative; N, normal; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MSCV, mean sphered corpuscular volume; MRV, mean reticulocyte volume; Ret, haematoцит; RDW, red cell volume distribution width; TBIL, total bilirubin; IBIL, indirect bilirubin; PK, pyruvate kinase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
Figure 1. Morphology of peripheral blood erythrocytes (Wright-Giemsa, magnification, x400). (A) Proband-mature erythrocytes varied slightly in size, were weakly stained in some areas, and were spherical, target-shaped, or polychromatic. (B) Father; (C) older sister; (D) mother-no obvious abnormalities by peripheral blood smears.

Figure 2. Sequencing results of solute carrier family 4 member 1 (Diego blood group) (SLC4A1) exon 3. Base substitutions occurred in exon 3 of SLC4A1: c.113 A>C, Asp 38 Ala. Arrows show the mutation sites. (A) Proband-homozygous mutation; (B) father-homozygous mutation; (C) older sister-heterozygous mutation; (D) mother-normal.
Li et al. (17) reported the results of HS disease-associated laboratory tests for three HS patients combined with β-thalassaemia. Weakly stained targeted erythrocytes and hyperchromatic spherical erythrocytes coexisted in the peripheral blood of the three patients. In the family survey, abnormal parameters were detected, and erythrocytes were hyperchromatic with spherical changes in the father or mother of the three patients; moreover, erythrocytes in their target shape were weakly stained in the other one of the three parents. Pathological changes of HS include erythrocyte membrane surface area loss and cell spheroidisation, whereas pathological changes of thalassaemia include insufficient cell content, a relatively enlarged erythrocyte membrane surface area and cellular targeting. Therefore, with regard to the membrane surface area, these two opposite changes in haemolytic indices interfere with each other and neutralize the effect of each other. In our patient, peripheral blood cell tests demonstrated two kinds of abnormal erythrocytes, but these abnormalities were not observed in his parents or older sister. Erythrocyte osmotic fragility increases in patients with HS, but decreases in patients with thalassaemia. Thus, the erythrocyte osmotic fragility did not dramatically alter in our patient (AGLT 50 >290s). This indicated that a single haemolytic factor cannot confirm this disease, and pedigree investigation is necessary.

The results of genetic diagnosis suggested that the gene deletion mutation of the child with thalassaemia was derived from his mother. -α 3.7 deletion thalassaemia-2 is common in carriers of α-thalassaemia, who do not present with clinical symptoms9. Nevertheless, since birth, the patient was affected by significant anaemia and jaundice. Moreover, Hb, MSCV, and MRV were markedly reduced. These factors and results from the related haemolytic anaemia test suggested that the patient probably had α-thalassaemia combined with HS. We detected two mutation sites of the SLC4A1 gene: Exon 3 c.113A>C (Asp 38 Ala) and intron 7 c.609+86G>A. Gene mutations affect mRNA expression levels or protein stability. Furthermore, mutations in exons often lead to amino acid changes, affect protein function, and cause membrane protein defects (21,22). In some HS patients, intron mutations affected RNA cleavage resulting in RNA instability and degradation (23). Although the patient's mother harboured an abnormal thalassaemia gene and his father had the identical homozygous gene mutation of HS, they did not present with significant clinical symptoms or abnormal hematologic results. The patient suffered from two genetic defects. Compared with a patient with α-thalassaemia or HS alone, his symptoms were complicated. Because of the coexistence of α-thalassaemia, excessive β-globin chains caused by an imbalance in erythrocytes by HS can gather on cell membranes and link with membrane proteins, which stiffen the membrane and reduce deformability. Thus, these cells can easily be injured by oxidation, resulting in increased membrane permeability and intracellular K+ leakage. The influx of Na+ and water into cells lead to erythrocyte swelling, rupture, and eventually haemolysis. These symptoms may become aggravated if the patient suffers from infection or is administered oxidising drugs.

In summary, the diagnosis of thalassaemia combined with HS is difficult because of the interference of Hb synthesis disorders and erythrocyte membrane defects with regard to the relevant laboratory results. When clinical manifestations and laboratory results cannot be explained by haemolytic...
anaemia alone, the possibility of combination with another haemolytic anaemia should be considered. Currently, many advanced techniques are of help, and it is necessary to perform pedigree investigation and genetic analysis for accurate diagnosis.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (no. 81360263).

References

1. Pan HF, Long GF, Li Q, Feng YN, Lei ZY, Wei HW, Huang YY, Huang JH, Lin N, Xu QQ, et al: Current status of thalassaemia in minority populations in Guangxi, China. Clin Genet 71: 419-426, 2007.
2. Zheng CG, Liu M, Du J, Chen K, Yang Y and Yang Z: Molecular spectrum of α- and β-globin gene mutations detected in the population of Guangxi Zhuang Autonomous Region, People's Republic of China. Hemoglobin 35: 28-39, 2011.
3. Xiong F, Sun M, Zhang X, Cai R, Zhou Y, Lou J, Zeng L, Sun Q, Xiao Q, Shang X, et al: Molecular epidemiological survey of haemoglobinopathies in the Guangxi Zhuang Autonomous Region of southern China. Clin Genet 78: 139-148, 2010.
4. Li J, Huang Z, Xu Y, Zhou H, Han F, Gong S and Wan S: Compound heterozygote factor and clinical significance of hemolysis system analysis in the diagnosis of congenital hemolytic anaemia: Etiological analysis of 506 cases of anaemia and jaundice. J Clin Hematol 18: 204-206, 2005.
5. Perrotta S, Gallagher PG and Mohandas N: Hereditary spherocytosis. Lancet 372: 1411-1426, 2008.
6. Shang H, Wang YS and Shen ZY: National Guide to Clinical Laboratory Procedures.4th edition. People's Medical Publishing House, Beijing, 2014.
7. Xu XM, Zhang XH and Chen LL: Guidelines for Thalassemia Prevention and Control Programme. People's Military Medical Press, Beijing, 2011.
8. Higgs DR, Engel JD and Stamatoyannopoulos G: Thalassaemia. Lancet 379: 373-383, 2012.
9. Harteveld CL and Higgs DR: Alpha-thalassaemia. Orphanet J Rare Dis 5: 13, 2010.
10. Piel FB and Weatherall DJ: The α-thalassemias. N Engl J Med 371: 1908-1916, 2014.
11. An X and Mohandas N: Disorders of red cell membrane. Br J Haematol 141: 367-375, 2008.
12. Bolton-Maggs PH, Langer JC, Iolascon A, Tittensor P and King MJ: General Haematology Task Force of the British Committee for Standards in Haematology: Guidelines for the diagnosis and management of hereditary spherocytosis-2011 update. Br J Haematol 156: 37-49, 2012.
13. Maciag M, Adamowicz-Salach A, Siwicka A, Szychalska J and Burzynska B: The use of real-time PCR technique in the detection of novel protein 4.2 gene mutations that coexist with thalassaemia alpha in a single patient. Eur J Haematol 83: 373-377, 2009.
14. Uysal Z, Yildirmak Y, Akar N, Basak N and Cin S: Alpha-Thalassemia and hereditary spherocytosis in the same patient: The interaction of two diseases. Pediatr Hematol Oncol 15: 271-276, 1998.
15. Uysal ZL, Akar N, Cin S, Ekici F and Başak N: Homozygous β-thalassemia (FCS8-AA) and hereditary spherocytosis in the same patient. Turk J Haematol 18: 137-141, 2001.
16. Akar N and Gökçe H: Red blood cell indexes in patients with hereditary spherocytosis and beta-thalassemia combination. Pediatr Hematol Oncol 19: 569-573, 2002.
17. Li J, Chen L, Huang Z, Fang C and Cui B: Differential diagnosis of hereditary spherocytosis associated with beta thalassemia. J Diagn Concepts Pract 9: 225-228, 2010.
18. Broséus J, Visomblain B, Guy J, Maynadie M and Girodon F: Evaluation of mean spherical corpuscular volume for predicting hereditary spherocytosis. Int J Lab Hematol 32: 519-523, 2010.
19. Liao L, Deng ZF, Qiu YL, Chen P, Chen WQ and Lin FQ: Values of mean cell volume and mean spherical cell volume can differentiate hereditary spherocytosis and thalassemia. Hematology 19: 393-396, 2014.
20. Xu Y, Yang W, Liao L, Deng Z, Qiu Y, Chen W and Lin F: Mean reticulocyte volume: A specific parameter to screen for hereditary spherocytosis. Eur J Haematol 96: 170-174, 2016.
21. Boguslaw ska DM, Heger E, Baldy-Chudzik K, Zagulski M, Maciejewska M, Li kwizar A and Sikorski AF: (AC)n microsatellite polymorphism and 14-nucleotide deletion in exon 42 ankryn-1 gene in several families with hereditary spherocytosis in a population of South-Western Poland. Ann Hematol 85: 337-339, 2006.
22. Ozcan R, Jarolim P, Lux SE, Ungewickell E and Eber SW: Simultaneous (AC)n microsatellite polymorphism analysis and single-stranded conformation polymorphism screening is an efficient strategy for detecting anklyn-1 mutations in dominant hereditary spherocytosis. Br J Haematol 122: 669-677, 2009.
23. Edelman EJ, Maksimova Y, Duru F, Altay C and Gallagher PG: A complex splicing defect associated with homozygous ankryn-deficient hereditary spherocytosis. Blood 109: 5491-5493, 2007.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.